IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Lisa A. Neuhold et al.

Application No.: 09/717,450 Art Unit: 1632

Filed: November 20, 2000 Examiner: M. C. Wilson

For: TRANSGENIC ANIMAL MODEL FOR DEGENERATIVE DISEASES OF CARTILAGE

DECLARATION OF DR. ROGER ASKEW UNDER 37 C.F.R. § 1.132

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, ROGER ASKEW, do hereby declare and state the following:

1. I, Roger Askew, am a citizen of the United States, and I am more than twenty-one years of age.

2. I make this declaration in support of the above-identified application ("the '450 application").

3. I presently hold the position of Director of Molecular Genetics at Wyeth Research, Andover, Massachusetts, and have held this position for three years.

4. My qualifications as a scientist, and in particular in the field of transgenic animals and gene-targeting, are set forth on the copy of my curriculum vitae, which is attached as Exhibit A..

5. I have read and am familiar with the specification of the '450 application as filed, the Final Office Action mailed November 11, 2004 in connection with this application, Applicants' Response to Final Action mailed January 2, 2005, the Declaration of Lisa A. Neuhold, Ph.D. under 37 C.F.R. 1.132 mailed on April 6, 1999 in connection with the parent of the '450 application (U.S. Serial No. 08/994,689; "the '689 application"), and the Second Declaration of Lisa A. Neuhold, Ph.D. under 37 C.F.R. 1.132 mailed on August 31, 2000 in connection with the '689 application.

6. It is my understanding that the Examiner believes that the '450 application does not teach chondrocyte-specific promoters and that one of ordinary skilled molecular biologist would not know how to use such promoters in the context of the '450 application's invention. The following paragraphs (7-8) describe sections of the '450 application and references demonstrate that chondrocyte-specific promoters are adequately described in the '450 application and were known by ordinary skilled molecular biologists at the time the '689 application was filed (1997).

7. There is a description of promoters that direct transcription in joint tissues, such as chondrocyte-specific promoters, *i.e.*, that provide spatial control of expression, in the '450 application (see for example, page 15, line 19 to page 16, line 6). The application discloses that expression of a matrix decoding enzyme (MDE) in chondrocytes, which are the cells found in

articular cartilage of the joint, results in localized degradation of extracellular matrix proteins. Having established this principle with a working example (the Type II collagen promoter), one of ordinary skill in the art would recognize that joint (*i.e.*, chondrocyte) tissue-specific expression of an MDE, particularly a collagen II-degrading MMP, would yield the desired joint degradation

8. As evidence that chondrocyte-specific promoters were known by those of ordinary skill in the art at the time of the present invention, I provide and discuss references dating from the approximate time of the invention exemplifying the highly characterized nature of other chondrocyte-specific promoters. The first reference, Pirok *et al.*, Structural and Functional Analysis of the Chick Chondroitin Sulfate Proteoglycan (Aggrecan) Promoter and Enhancer Region. *The Journal of Biological Chemistry*. Vol. 272, No. 17, pp. 11566-11574, 1997 (attached as Exhibit B) discloses mapping of 5' segments (enhancer and silencer, *i.e.* promoter, elements) of a cartilage-specific gene: the chondroitin sulfate proteoglycan (CSPG; Aggrecan) gene. These promoter elements are responsible for the chondrocyte-specific expression of the CSPG gene. Another reference, which was received by the journal in which it is published in May 1998 and thus clearly represents work done in 1997, (Lefebvre *et al.*, A New Long Form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene, *The Embo Journal*. Vol. 17, No. 19, pp. 5718-5733, 1998; "Lefebvre"; attached as Exhibit C), discloses cooperation of transcription factors Sox5 and Sox6 in providing chondrocyte-specific expression of the *Col2a1* gene. Specifically, this reference describes the elements of at least two promoters, the *Col2a1* and *Col11a2* promoters, which lead to chondrocyte-specific gene expression (see, e.g., p. 5719, col. 1 first and second full paragraphs of Lefebvre). Accordingly, not only do these references describe chondrocyte-specific promoters known in the art at the time of the invention, but they also describe the characterization of the molecular features of these promoters.

In conclusion, both of the references described chondrocyte-specific promoters known in the art at the time of the present invention.

9. Additionally, it would be routine for one of ordinary skill in the art to identify the promoter(s) responsible for chondrocyte-specific expression, e.g. promoter of the chondrocyte-specific genes in references above. For example, simple β-galactosidase expression experiments would demonstrate whether a promoter were a chondrocyte-specific promoter.

10. It is my understanding that the Examiner believes that the '450 application does not teach transgenic non-human mammals other than mice and that an ordinary skilled molecular biologist would not know how to use such transgenic non-human mammals, particularly rats, in the context of the '450 application's invention. In coming to this conclusion, the Examiner cites several references (e.g. Mullins *et al.*, Nature, 1990, 344:541-544; Hammer *et al.*, Cell, 1990, 63:1099-1112; Mullins *et al.*, Hypertension, 1993, 22(4):630-633), asserting that these references demonstrate the unpredictability of developing transgenics. However, none of the references pointed to by the Examiner are directed to the same system targeted in the transgenic mammal (e.g., rat) of the present invention. The instant invention uses a constitutively enzymatically active human matrix metalloproteinase to cleave type II collagen. Degradation of type II collagen is not highly species dependent because Type II collagen is highly conserved between species. Degradation of type II collagen is not as complicated as phenotypes of references cited by Examiner (e.g. hypertension: see Mullins *et al.*, Nature, 1990, 344:541-544 and Mullins *et al.*, Hypertension, 1993, 22(4):630-633). Thus, success of the transgenic mouse, as is demonstrated in this application, is highly predictive of success in the transgenic rat.

11. It is also my understanding that the Examiner believes that it would require one of skill undue experimentation to ensure that that any transgenic non-human mammal would express MMP to a level sufficient to cause Type II collagen degradation in the joints. In my sixteen years of experience in the trangenics field, it would not require undue experimentation to translate success of a transgenic mouse into success of a transgenic rat. In my laboratory we have successfully translated several transgenic mouse models into rat models. For example, we have successfully extrapolated a mouse ALS model to the rat using the exact same transgene used to generate the mouse.

12. I further declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true. I further declare that these statements are made with the knowledge that the willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States code, and that such willful false statements may jeopardize the validity of the instant application or of any patent issued thereupon.

Respectfully submitted,

9-02-05

DATE

Roger Askew, Ph.D.

Curriculum Vitae

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Education:

1989 Ph.D., Department of Molecular Biology and Biochemistry, Wesleyan University, Middletown, CT.

1980 B.S., Biology, University of Connecticut, Storrs, CT.

Positions:

2003 - current Associate Director, Molecular Genetics, Department of Applied Genomics, Wyeth Research, Andover, MA

2001- 2003 Principle Scientist I, Department of Molecular Genetics, Wyeth Research, Andover, MA

1999 - 2001 Senior Research Scientist II, Department of Molecular Genetics, Wyeth Research, Andover, MA

1996- 1999 Senior Research Scientist, Department of Molecular Genetics, Wyeth-Ayerst Research, Princeton, NJ

1994 - 1996 Senior Scientist, Department of Molecular Genetics, Wyeth-Ayerst Research, Princeton, NJ

1992-1994 Research Associate, *Program of Excellence in Molecular Biology of Heart and Lung*, Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, Cincinnati, OH.

1989-92 Postdoctoral Assistant, Dr. Jerry B Lingrel, *Program of Excellence in Molecular Biology of Heart and Lung*, Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, Cincinnati, OH.

1984-89 Graduate student, Department of Molecular Biology and Biochemistry, Wesleyan University, Middletown, CT.

Invited Seminars:

2002 "Evaluation of a conditional knock out gene trapping strategy "
Second International Gene Workshop,
University of Frankfurt Medical School Frankfurt, Germany

2001 "Site directed transgenesis by recombinase driven insertion"
First International Gene Trap Workshop, Mount Sinai Hospital, Toronto, Canada

1999 "Phenotypic analysis of Estrogen Receptor-deficient mice"
Brekenridge Workshop on Steroid Receptors in Brain Function, Brekenridge, Colorado

1994 "Interaction of the Cardiac Glycoside Receptor with it's Ligand"
Program of Excellence Meeting (MIT, UCinci., UCSF), Santa Barbara, CA

1993 "Site directed Point Mutations in Embryonic Stem Cells: a Gene Targeting Tag-and-Exchange Strategy"
Department of Molecular Genetics, University of Cincinnati, Cincinnati,OH

1993 "Site directed Point Mutations in Embryonic Stem Cells: a Gene Targeting Tag-and-Exchange Strategy"
Division of Nephrology, Indiana University Medical School, Indianapolis, IN

1993 "Targeting Point Mutations in Embryonic Stems Cells by Sequential replacement"
Institute for Developmental Biology, Children's Hospital, Cincinnati, OH

Honors and Awards:

1992 American Heart Association , Ohio Affiliate Grant-In-Aid.
1988 Sigma Xi grant in aid of research award.
1988 Peterson Prize for excellence in biochemistry.
1987 Elected to membership in Sigma Xi.
1986 Sigma Xi grant in aid of research award.
1984-89 Full Graduate Tuition Fellowship, Wesleyan University.

Publications:

Glasson SS. Askew R. Sheppard B. Carito BA. Blanchet T. Ma HL. Flannery CR. Peluso D. Kanki K. Yang Z. Majumdar MK. Morris EA. Deletion of active ADAMTS5 prevents cartilage degradation in a murine model of osteoarthritis. Nature 434(7033):644-8, 2005 Mar .

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Lingrel JB, Van Huyesse J, Jewell-Motz B, Schultheis P, Wallick ET, O'Brien W, and Askew GR: Na,K-ATPase: Cardiac Glycoside Binding and Functional Importance of Negatively Charged Amino Acids of Transmembrane Regions. Kidney International, 1994

Lingrel JB, Van Huysse J, Obrien W, Jewel-Motz E, Askew GR, and Schultheis P: Structure-function studies of the Na,K-ATPase. In: Bamberg E, Schoner W, editors. The Sodium Pump. Springer, New York:Darnstatd and Steinkopff :276-286, 1994.

Askew GR, Doetschman T and Lingrel JB: Site-directed point mutations in embryonic stem cells: a gene targeting tag-and-exchange strategy. *Mol Cell Biol* 13:4115-4124, 1993.

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Abstracts:

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Yogendra Kharode, Paula Green, James Marzolf, Weiguang Zhao, Roger Askew, Paul Yavorsky and Frederick Bex. Alteration in bone density of mice due to heterozygous inactivation of LRP6. American Society for Bone and Mineral Research, 25th annual, 2003

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Askew GR and Lingrel JB: Gene modification of the α2 Na,K-ATPase gene in murine embryonic stem cells. Presented at the Gordon Conference on Molecular Genetics, 1991.

Patents:

Conditional knockout method for gene trapping and gene targeting using an inducible gene silencer
R Askew, M Barton and K Kanki. US Patent application (filed May 2003).

Case number AM100651

Knock in transgenic mammal containing a non-functional n-terminus of k_V beta 1.1 subunit

This application claims priority from a copending provisional application serial number 60/308,485, filed on July 27, 2001, the entire disclosure of which is hereby incorporated by reference.

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Structural and Functional Analysis of the Chick Chondroitin Sulfate Proteoglycan (Aggrecan) Promoter and Enhancer Region*

(Received for publication, September 24, 1996, and in revised form, February 5, 1997)

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Aggrecan is a large chondroitin sulfate proteoglycan, the expression of which is both tissue-specific and developmentally regulated. Here we report the cloning and sequencing of the 1.8-kilobase genomic 5′ flanking sequence of the chick aggrecan gene and provide a functional and structural characterization of its promoter and enhancer region. Sequence analysis reveals potential Sp1, AP2, and NF-I related sites, as well as several putative transcription factor binding sites, including the cartilage-associated silencers CHS1 and CHS2. A number of these transcription factor binding motifs are embedded in a sequence flanked by prominent inverted repeats. Although lacking a classic TATA box, there are two instances in the 1.8-kb genomic fragment of TATA-like TCTAA sequences, as have been defined previously in other promoter regions. Primer extension and S1 protection analyses reveal three major transcription start sites, also located between the inverted repeats. Transient transfections of chick sternal chondrocytes and fibroblasts with reporter plasmids bearing progressively reduced portions of the aggrecan promoter region allowed mapping of chondrocyte-specific transcription enhancer and silencer elements that are consistent with the sequence analysis. These findings suggest the importance of this regulatory region in the tissue-specific expression of the chick aggrecan gene.

During development, the extracellular matrix is a complex dynamic structure, the components and organization of which help to establish the requisite position and state of differentiation. The large chondroitin sulfate proteoglycan (CSPG),[1] aggrecan, has been localized predominantly to skeletal tissue and is considered to be a hallmark of cartilage differentiation. In chick cartilage, aggrecan expression begins at embryonic day 5 in limb rudiments, continues through the entire period of chondrocyte development, and remains a biochemical marker of the cartilage phenotype thereafter. In very early embryos, aggrecan is expressed in the notochord as early as stage 16, long before chondrogenesis occurs (1).

We have extensively studied the properties and expression of aggrecan from embryonic chick cartilage. These studies include synthesis and processing (2–5), structural analysis via peptide sequencing to elucidate glycosylation motifs, and a consensus sequence for *O*-xylosylation and mapping of the S103L monoclonal antibody epitope (6–10). Moreover, we have conducted molecular analysis to construct the composite sequence of chick cartilage CSPG from overlapping cDNAs and to identify a defect in the aggrecan gene associated with the chondrodystrophy, nanomelia (9, 11).

This sequence, obtained from 10-day-old chick embryos, has 6464 nucleotides that include an open reading frame encoding 2109 amino acids and 16 nucleotides of the first untranslated exon (11). Another chick aggrecan cDNA sequence, obtained from embryonic chick brain, was 6597 nt in length, including 265 nt of 5′-untranslated exon sequence (12). Using chick CSPG cDNA probes, we subsequently isolated genomic clones containing exons encoding the chick CSPG core protein. The two 5′ globular domains, G1 and G2, are encoded by four and three exons, respectively, and the interglobular domain is encoded by a single exon. The chondroitin sulfate attachment domain is encoded by the largest exon, 3216 bp, which is approximately 50% of the total coding sequence. These data reveal that the chick CSPG gene contains at least 18 exons spanning more than 30 kb. No evidence was obtained for multiple genes for aggrecan in the chick genome. Elucidation of the genomic organization of chick aggrecan has allowed for a more thorough comparison with the mammalian aggrecans, as well as the avian and mammalian link proteins, with respect to origin and mechanisms of divergence. A summary of this work was published recently (13).

We have also found that aggrecan is developmentally expressed, *in ovo* and in limb bud cultures, on both protein and mRNA levels in a pattern commensurate with the onset of chondrogenesis. The modulation of expression of this cartilage-specific CSPG and type II collagen mRNA in stage 24 limb bud mesenchyme cells cultured in high density was examined under conditions that promote chondrogenesis *in vitro* (14) and mimics the same process in limb development *in ovo*. Morphologically, mesenchymal proliferation ceases by day 2, condensation occurs first in the formation of aggregates by days 4–5, and then of overt nodules by days 6–8, concomitant with cellular differentiation and production of matrix. Quantitatively, a 50-fold increase in aggrecan mRNA occurs from day 2 (when first detected) to day 6, followed by a slight decline (about 2-fold) by day 8 when the message reaches a plateau thereafter (15). This same pattern is observed immunologically, using the monoclonal antibody S103L, which is specific to the aggrecan protein. These studies indicate that during limb development

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U83593.

‖ Deceased.

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[1] The abbreviations used are: CSPG, chondroitin sulfate proteoglycan; nt, nucleotide(s); kb, kilobase(s); bp, base pair(s); SP, signal peptide; PCR, polymerase chain reaction.

the expression of these two differentiation-specific proteins are stringently controlled until the establishment of the cartilage phenotype. Thereafter, aggrecan continues to be synthesized and deposited in the extracellular matrix, perhaps to effect a decrease in cell adhesion necessary for maintenance of the chondrogenic state.

Concurrent with studies of mechanisms that control the temporal-spatial aspects of cartilage differentiation are structural and functional analyses of expression of the differentiation-specific products of the extracellular matrix. For instance, significant work has been done to understand the tissue-specific expression of collagen genes and the mechanisms that regulate their distinct transcriptional programs (16–18). In contrast, there have been no studies of the transcriptional regulation of the aggrecan gene that examine its tissue-specific expression during development. Mouse aggrecan has been cloned; however, no functional analysis has been performed to examine its tissue specificity (19). A preliminary characterization of the rat aggrecan promoter has also appeared, describing a 120-bp sequence containing transcription start sites (20). It is not clear whether this 120-bp genomic fragment contains tissue-specific control elements, because the 5′ promoter/enhancer region is probably larger or may contain additional regulatory elements. The same report described promoter assays on a larger isolate containing an additional 520 bp of 5′ flanking sequence, but the sequence data were not presented.

Therefore, to begin to elucidate the mechanisms that govern aggrecan expression in chondrocytes, we have cloned the promoter region of the embryonic chick S103L-reactive CSPG (aggrecan). The aim of the present study was to identify and characterize the cell- and stage-specific elements in the 5′ genomic flanking region of the aggrecan gene, which could regulate the expression of this extracellular macromolecule during embryonic development.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were made with an Applied Biosystems 3808 DNA synthesizer. Reagents for biochemical and molecular cloning experiments were of the highest quality available from commercial vendors. Restriction endonucleases were from New England Biolabs unless otherwise stated. T4 DNA ligase, T4 kinase, S1 nuclease, avian myeloblastosis virus reverse transcriptase, and Klenow polymerase were from Promega. Taq polymerase was from Perkin-Elmer. A chick genomic library was purchased from CLONTECH Laboratories.

Preparation of Probe and Screening of Chick Genomic Library—A chick aggrecan cDNA fragment comprising 260 bp of the 5′-untranslated exon plus 56 bp of the signal peptide (SP) exon was obtained via PCR from the previously reported cDNA, clone 1 (11). Because the template clone was inserted in pGEM-4Z, the upstream primer was the SP6 promoter primer (Promega); the downstream primer was a 17-mer, 5′-CTGTGGTGATGGCTTGC-3′, from the antisense strand of the SP exon. The probe was then purified by low-melting-point agarose gel electrophoresis and labeled with ^{32}P using a Multiprime DNA labeling system and [α-^{32}P]dCTP purchased from Amersham Corp. Approximately 50,000 independent members of the chick genomic library were screened. The chick genomic library was plated, and nitrocellulose plaque-lifts were prepared and probed by hybridization according to standard methods (21). Positive plaques were picked, then re-plated, and screened as above for two or three rounds until the plaques were purified.

Isolation of Chick Aggrecan Genomic Clones—The screening yielded a 14-kb genomic fragment (Fig. 1*B*). Phage DNA was purified from plate lysates (21). Isolates from the library screening were subcloned into the vector pGEM-4Z by standard methods (21). Southern blot analysis using the same aggrecan untranslated exon probe identified an approximately 1.8-kb *Bgl*II-*Bbs*I genomic fragment that was subcloned into pGEM-4Z. Initial sequencing with the T7 promoter primer (Promega) revealed that one end of the subclone had a sequence identical to the 5′ 145 bp of a previously published S103L-CSPG cDNA sequence (12), with the exception of three dA residues that were not present in the cDNA sequence. The genomic clone has a tract of 21 dAs where the cDNA has a stretch of 18 dAs. This likely reflects an error arising during library generation because the flanking sequences are identical. The 1.8-kb insert was excised from pGEM-4Z by *Eco*RI-*Kpn*I digestion, treated with Klenow polymerase, and blunt-end ligated into the reporter vector pGL2-Basic (Promega), which had been linearized with the restriction enzyme *Nhe*I and treated with Klenow. The reporter vector pGL2-Basic does not contain any eukaryotic promoter or enhancer elements. Sequences to be assayed for promoter activity are inserted upstream (5′) of a luciferase gene. Plasmids were sequenced to find clones that had the insert positioned in the forward (+) and reverse (−) orientations (Fig. 2*C*). The forward orientation was defined as having the 1.8-kb insert ligated into the reporter vector pGL2-Basic with the same 5′-3′ orientation relative to the reporter gene as the native sequence in the genomic clone relative to the aggrecan gene. Constructs that contained the 1.8-kb genomic insert of the chick aggrecan gene were named Ag-1(+) and Ag-1(−).

Sequence Determination and Analysis—Dideoxynucleotide chain termination sequencing (22) of the *Bgl*II/*Bbs*I DNA fragments subcloned into pGEM-4Z plasmids was performed using the U. S. Biochemical Sequenase (version 2.0) system. Primers were T7 or SP6 promoter primers (Promega) or 18–20-mer oligonucleotides synthesized according to the obtained sequence. Multiple sequence determinations were made for each primer used. Ambiguities in sequencing were resolved by using a different polymerase (*e.g.* avian myeloblastosis virus reverse transcriptase), sequencing the complementary strand, or both. All residues were confirmed by at least two separate sequence determinations. DNA sequence analysis was performed using the Wisconsin Package (23). Searching for palindromic sequences was done using the program COMPARE to find inverted repeats by comparing the sequence to its own complement (24), and the results were displayed via the program DOTPLOT. Putative transcription factor binding sites were located with the program FINDPATTERNS using the pattern file tfsite.dat, which comprises the Transcription Factor Database (25).

Purification of DNA—Plated colonies were used to inoculate 5 ml of LB medium (21). The cells were grown overnight at 37 °C with vigorous shaking. The 5-ml culture was added to 400 ml of LB. The culture was shaken at 37 °C for at least 12 h, cells were harvested, and plasmid DNA was recovered using the QIAGEN Plasmid Maxiprep kit.

Synthesis of Deletion Constructs—The inserts for plasmid constructs 1300(+), 900(+), 500(+), and 500(−) were made by PCR using the Ag-1(+) construct as a template (Fig. 2, *A* and *B*, and Fig. 6*B*). *Xho*I sites were introduced at the end of the amplified fragments via the primers used. PCR fragments were purified using Qiaquick PCR Preps (QIAGEN) and digested with *Xho*I for 2 h. The fragments were gel purified and ligated into the *Xho*I site of the pGL2-Basic vector. Inserts A(+) to F(+) were made via PCR with Ag-1(+) as a template, and the primer oligonucleotides contained downstream *Bgl*II/*Sma*I and upstream *Kpn*I restriction enzyme cutting sites. The PCR fragments were gel purified, digested with *Bgl*II and *Kpn*I, and ligated directly into pGL2-Basic, producing the constructs A(+) to F(+) (Fig. 2, *A* and *B*, and Fig. 6*B*). The constructs A(−) to F(−) were made in the same fashion as above, except that each insert was digested with *Sma*I and *Kpn*I at the insert ends to ensure their opposite orientation in the pGL2-Basic vector relative to the A(+) to F(+) inserts (Fig. 2, *A* and *B*, and Fig. 6*B*). Sequencing of the various constructs was done to confirm the appropriate orientation of the inserts and exclude PCR artifacts.

Cell Cultures—Cultures of day-14 chick sternal chondrocytes were established according to the procedures described by Cahn *et al.* (26) and as modified by Campbell and Schwartz (3). Cultures of fibroblasts were established from skin of day-10 chick embryos following trypsinization (3). Cells were plated at an initial density of 1.5×10^6/100-mm tissue culture dishes (Falcon) in either F-12 medium (chondrocytes) or Dulbecco's modified Eagle's medium (fibroblasts) and supplemented with 10% fetal calf serum. The cells were permitted to attach to the dishes, and subsequent growth (2–3 days) was maintained by a complete change of the medium every 2 days (2). On the day of transfection, chondrocyte cultures were trypsinized, and single cells were suspended in F-12 medium, replated, and allowed to attach to the dishes for 3–4 h before treatment as described below.

Transfection—Standard methods were followed for transient calcium phosphate transfections (21). Duplicate plates containing approximately 5×10^6 cells (either chondrocytes or fibroblasts) received 20 pmol of a given plasmid construct to be assayed. Five μg of a β-galactosidase reporter plasmid were cotransfected with each experimental construct to correct for cell loss. Duplicate transfection sets were repeated three times, each time yielding similar results. The transfections were allowed to proceed for 36 h. The relative efficiency of transfecting the chondrocytes was approximately 13% that of transfecting the fibroblasts.

Cell Recovery and Assays—Reagents for the luciferase and β-galactosidase assays were purchased from Promega. Because both luciferase

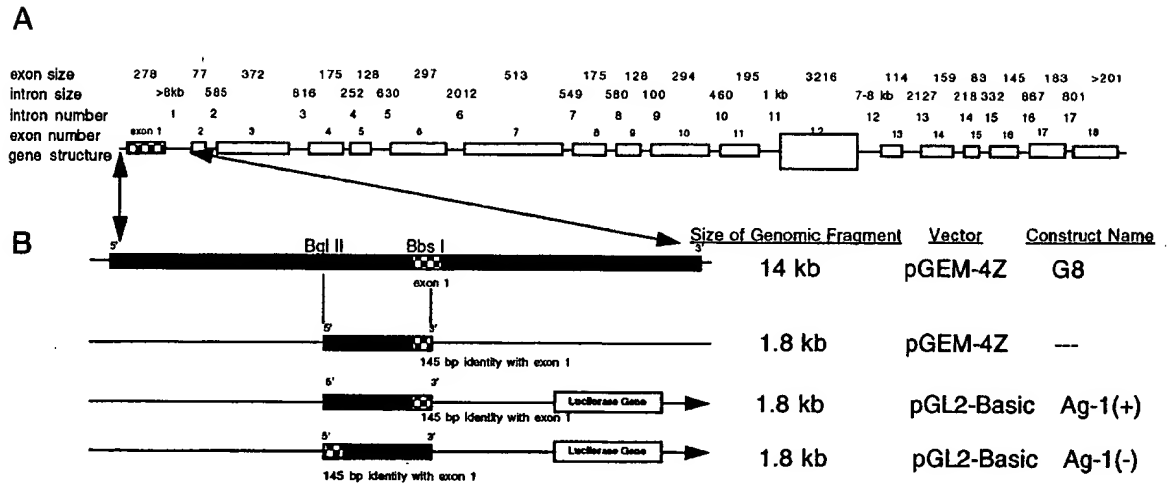

FIG. 1. **Chick aggrecan genomic structure and cloning strategy.** *A*, a schematic diagram of the genomic structure of the chick aggrecan gene. The names and sizes of the introns or exons are printed *above* the diagram. Exons are represented as *open boxes*, except the first untranslated exon, which is represented as a *checkered box*. Introns are represented as the *lines* connecting the *boxes*. The diagram is not to scale. *B*, the cloning strategy for the chick aggrecan promoter region. On the *right side* of the diagram is a chart that indicates the size, vector, and name used for each construct. As described under "Experimental Procedures," cDNA from the untranslated and signal peptide exons was used to screen a chick genomic library. The 14-kb genomic fragment obtained was subcloned into the vector pGEM-4Z and is represented as a *black rectangle* with the *checkered pattern* indicating the region of overlap with the first untranslated exon. The 14-kb fragment was digested with *Bgl*II and *Bbs*I, and the resultant 1.8-kb fragment was subcloned into the sequencing vector pGEM-4Z and the luciferase reporter vector pGL2-Basic, which does not contain a eukaryotic promoter region. Each orientation of the genomic inserts was confirmed by sequencing.

assays and β-galactosidase assays were performed, Promega's Reporter Lysis Buffer (RBL, E3971) was used to prevent the inhibition of β-galactosidase activity that occurs in buffers containing detergents such as Triton X-100. No deviations were made from the manufacturer's protocol for preparation of extracts from tissue culture cells. The enzymatic activity of luciferase was measured with a luminometer (Analytical Luminescence Laboratory, Monolight 1500). The enzymatic activity for β-galactosidase was measured with a microplate reader (Dynatech) at 409 nm. Standard deviations were determined for the six assays performed on duplicate plates within one experiment.

End-labeling of Probes for mRNA 5′ End Mapping—The Z2 or Z3 oligonucleotides (Z2, 5′-AATTCCCTGTGTGGTATTTCAGGTCCTTTCAGGC-3′, nt 193–226; Z3, 5′-GCAAGAGAGACCATCAAACTCCTGTCAGCCTCCT-3′, nt 68–101) for primer extension experiments or S1 analysis were end labeled using [γ-^{32}P]ATP and T4 DNA kinase according to standard protocols (21). Three ethanol precipitations were performed to remove the residual [γ-^{32}P]ATP from the labeled oligonucleotides.

S1 Analysis of mRNA Using Single-stranded DNA Probes—Established methods were used to perform S1 analysis (27). Single-stranded probes were made from the double-stranded 900(+) and D(+) plasmids. Plasmids were alkali-denatured, and a ^{32}P-5′-end-labeled oligonucleotide primer, Z2 or Z3, was annealed to the template, 900(+) or D(+), and extended with Klenow (Promega). Probes were cut to the appropriate 5′ length by digestion with restriction enzyme *Kpn*I. The single-stranded probes were separated from the template DNA by alkaline low-melting-point agarose electrophoresis, and radiolabeled bands were cut out and purified by phenol extraction and ethanol precipitation (21). Approximately 5000 cpm of probe was hybridized to 25 μg of total RNA from day-14 chick sternal chondrocytes. The hybridization occurred at 55 °C for 12 h in an aqueous hybridization solution (21). The resultant RNA:DNA hybrid was digested with 200 units of S1 nuclease for 60 min. The products were electrophoresed in 6% polyacrylamide sequencing gels.

Primer Extension—Approximately 5000 cpm of labeled Z2 or Z3 probe was hybridized to 25 μg of RNA derived from day-1 chick sternal chondrocytes. Hybridization was done in S1 hybridization solution for 12 h at 30 °C (21). Extended products were produced by treating the hybrid RNA:primer with 40 units of avian myeloblastosis virus reverse transcriptase (Promega). Products were extracted in phenol/chloroform, precipitated in ethanol, and electrophoresed on 6% polyacrylamide sequencing gels.

RESULTS

Structural Analysis of the 5′ Portion of the Chick Aggrecan Gene—To guide functional studies, the complete 1.8-kb Ag-1 sequence was determined and found to comprise 1875 bp (Fig. 3). Examination of the sequence revealed the lack of a classical TATA box or CCAAT box. When the Ag-1 fragment was analyzed for transcription factor binding sequences, it was found that at least 202 potential sites were present, including putative AP2 and Sp1 binding sites. The relative positions of some of these eukaryotic transcription factor-associated sequences are indicated in Fig. 3. The numbering of the sequence is relative to the most upstream transcription start site (as detailed below). The Ag-1 sequence was also compared with known promoter sequences in the eukaryotic promoter data base (EPD) using the National Center for Biotechnology Information BLAST server (25), and no extensive identity with other promoter sequences was found. However, tracts of multiple dA and dT residues, analogous to those found in Ag-1 in the ranges 250 to 280 and −144 to −78, respectively, were seen to occur in many other described promoter regions. These dA and dT tracts, in particular the dT_{16} from −87 to −78 and the dA_{21} from 250 to 270, constitute an inverse repeat or palindrome with the potential to give rise to a pair of large stem-and-loop structures or a cruciform structure (28). Hence, additional analyses were performed on the Ag-1 sequence with the aim of detecting other, less obvious, palindromic sequences.

The Ag-1 sequence from positions −300 to 340 was analyzed by comparison to its own reverse complement sequence with the Wisconsin Package program COMPARE. The dot plot reveals a widely spaced pair of inverted repeats centered around −100 and 250, corresponding to the dT and dA tracts, separated by over 300 bp. However, no other potential secondary structures of comparable scale are seen in this sequence with the window/stringency parameters used in this analysis; a few less prominent repeat pairs occur in the downstream third of the sequence. Interestingly, the putative Sp1, AP2, and TFII sites in addition to other potential factor-specific sequences, as well as all three of the mapped start sites, lie in the putative loop portion of this potential structure. Such secondary structures, in addition to potential transcription factor binding sites, may be involved in mechanisms by which the aggrecan message is developmentally regulated.

Determination of Transcription Starting Sites—Two methods, S1 analysis and primer extension, were used to locate

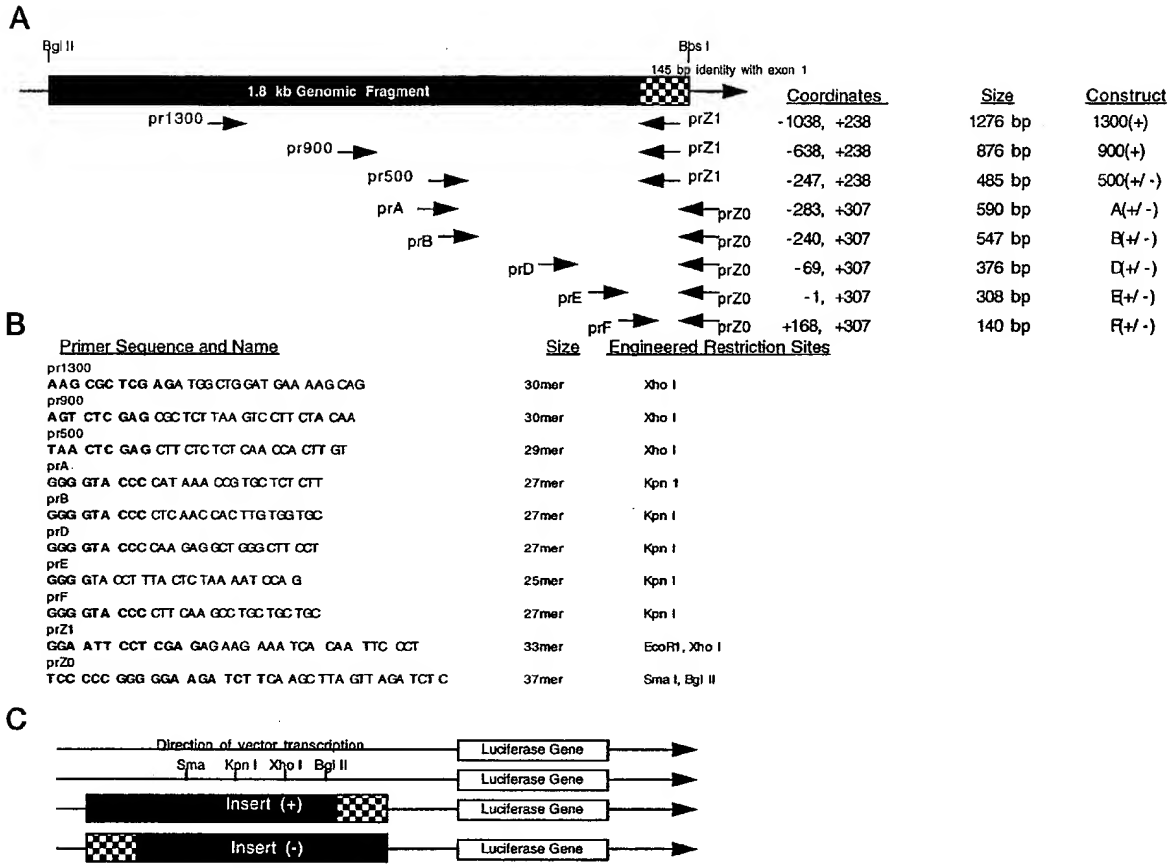

FIG. 2. **Schematic representation of the pGL2-Basic vector and the primers used to synthesize the deletion constructs.** *A,* the 1.8 genomic fragment Ag-1. The *checkered pattern* represents the 145-bp identity with the aggrecan cDNA. Above the diagram are the restriction enzymes used to excise this genomic fragment from the clone G8. Below are shown the names and relative positions of the primers used to make the deletion constructs. To the *right side* of the diagram is a chart that indicates the coordinates that the primer pairs span, the size of the resultant PCR product, and the names of the constructs. *B, below* the diagram is a list of all of the primers used to make deletion constructs with the Ag-1 sequence as a template. The *boldface* represents sequences not found in Ag-1 but added to engineer restriction enzyme cutting sites. Note that prZ0 resides in the vector pGL2-Basic and not in the Ag-1 sequence. *C,* a schematic representation of the pGL2-Basic vector and the relative positions of the restriction sites used in these experiments. The *arrow* represents the direction of transcription of the vector. The (+) and (−) orientations are defined as the positionings of the insert with respect to the luciferase gene in the same or reversed ways as it occurs with respect to the aggrecan coding sequence.

the sites where transcription of the aggrecan mRNA is initiated. Because the 5′-untranslated cDNA sequence previously reported by this laboratory (11, 12) overlaps with the 3′ end of the Ag-1 genomic isolate by 145 nucleotides, transcription initiation occurs still farther upstream in Ag-1. Templates used to generate single-stranded DNA probes for S1 analysis included the 900(+) and D(+) plasmid constructs, as represented in Fig. 4*C*. S1 analysis with the downstream primer Z2 yielded three major protected fragments: 226 bp, 187 bp, and a 69/70-bp doublet, corresponding to start sites at positions 1, 40, and 157–158 (Fig. 4*A, lanes 1* and *2*). Position 1 in Fig. 3 is defined as the farthest 5′ transcription starting site. These locations were obtained with probes generated from both the 900(+) and the D(+) constructs. The two upstream transcription start sites at positions 1 and 40 were confirmed with the downstream primer Z3-generated probes, again using the 900(+) and D(+) constructs as DNA templates (Fig. 4*B, lanes 4* and *5*). Z3-generated probes from the 900(+) and D(+) constructs gave protected fragments of 101 and 62 bp, respectively, confirming the position 1 and 40 transcription starting sites. The Z3 primer lies upstream of the 157/158 transcription starting site.

Primer extension experiments used the same antisense oligonucleotides, Z2 and Z3, as used in the S1 analyses. Primer extensions on RNA from cultured day-14 sternal chondrocytes gave products of the same sizes as the corresponding S1-protecting experiments, confirming the three transcription starting sites at positions 1, 40, and 157–158, as shown in Fig. 4, *A* and *B, lanes 3* and *6*. These results are represented schematically in Fig. 4*D*.

Functional Analysis of the Aggrecan Promoter Sequence—Transient transfections of day-14 chick embryo sternal chondrocytes with the construct Ag-1(+) (the forward orientation of the 1.8-kb insert in the promoter/enhancer-free pGL2-Basic reporter vector) revealed a plasmid dose-dependent level of luciferase expression (Fig. 5*A*), *i.e.* increasing concentrations of transfected construct produced increases in luciferase activity, establishing that the 1.8-kb region contains elements capable of promoter function. In subsequent experiments, constructs Ag-1(+) and Ag-1(−), in addition to pGL2-Basic vector with no insert, were transiently transfected into both 14 day-old chick sternal chondrocytes and, to examine tissue specificity, into 10 day-old chick embryo fibroblasts. In transfected chondrocytes, the construct Ag-1(+) produced a 45-fold increase in luciferase activity compared with the no-insert control (Fig. 5*B*), whereas transfected fibroblasts produced less than a 10-fold increase. Transfections with either the negative control pGL2-Basic vector with no insert or the Ag-1(−) construct resulted in much lower luciferase expression, with activity equivalent to background in both transfected chondrocytes and fibroblasts.

A series of constructs that progressively deleted the Ag-1(+) sequence was used to relate the locations of potential transcription factor binding sites and secondary structure to promoter

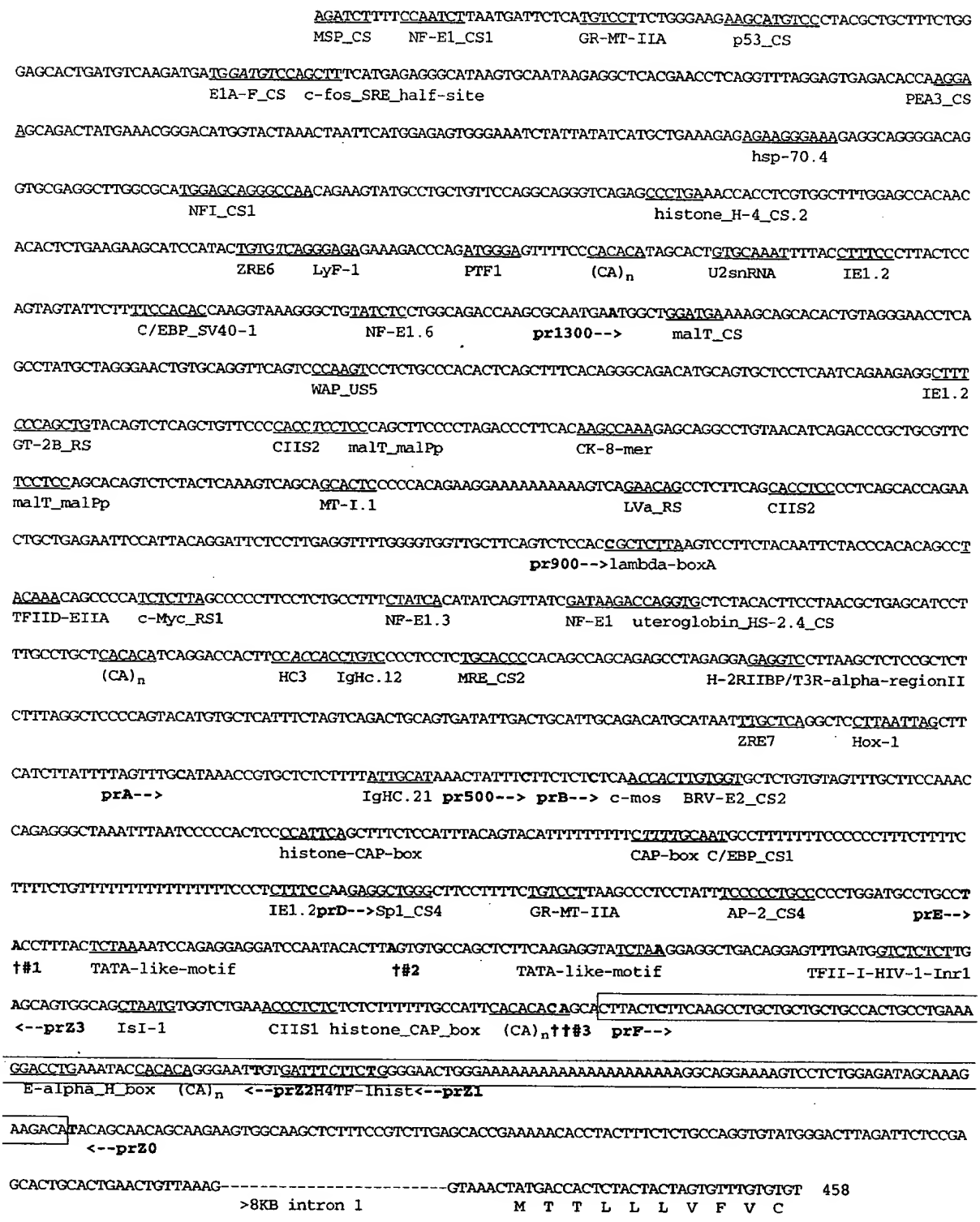

FIG. 3. **Nucleotide sequence and putative regulatory elements of the 5′ flanking region of the chick aggrecan gene.** The three major transcription start sites are indicated by the *daggers* followed by the respective number. Putative transcription factor binding sequences are *underlined,* and the GenBank names are printed *below* the *underlined sequence.* Overlapping binding sequences are in *italic* print. Sites were defined using the program FINDPATTERNS or from published papers. For clarity, only selected potential transcription factor binding sequences have been shown. Additionally, the 5′ region of primers designed to create the various deletion constructs are highlighted in *boldface* in the sequence, and below the sequence, the names of the primers are printed in *boldface* with *arrows* indicating the direction of their orientation. *Dashed lines* represent the >8 kb of intron that separates exon 2 (SP) from the first untranslated exon. The *boxed sequence* represents the region of the clone Ag-1 that overlaps with the cDNA sequence published previously. Note that position +307 is the *Bbs*I cutting site; thus, the Ag-1 sequence ends at this point.

function and tissue specificity. The constructs and transfection results are summarized in Fig. 6. The initial deletion removed approximately 500 bp from the upstream end of the Ag-1(+) construct, as well as a tract of 21 dA residues from the downstream end. The resulting construct, 1300(+), produced a modest increase in luciferase activity in chondrocytes *versus* that

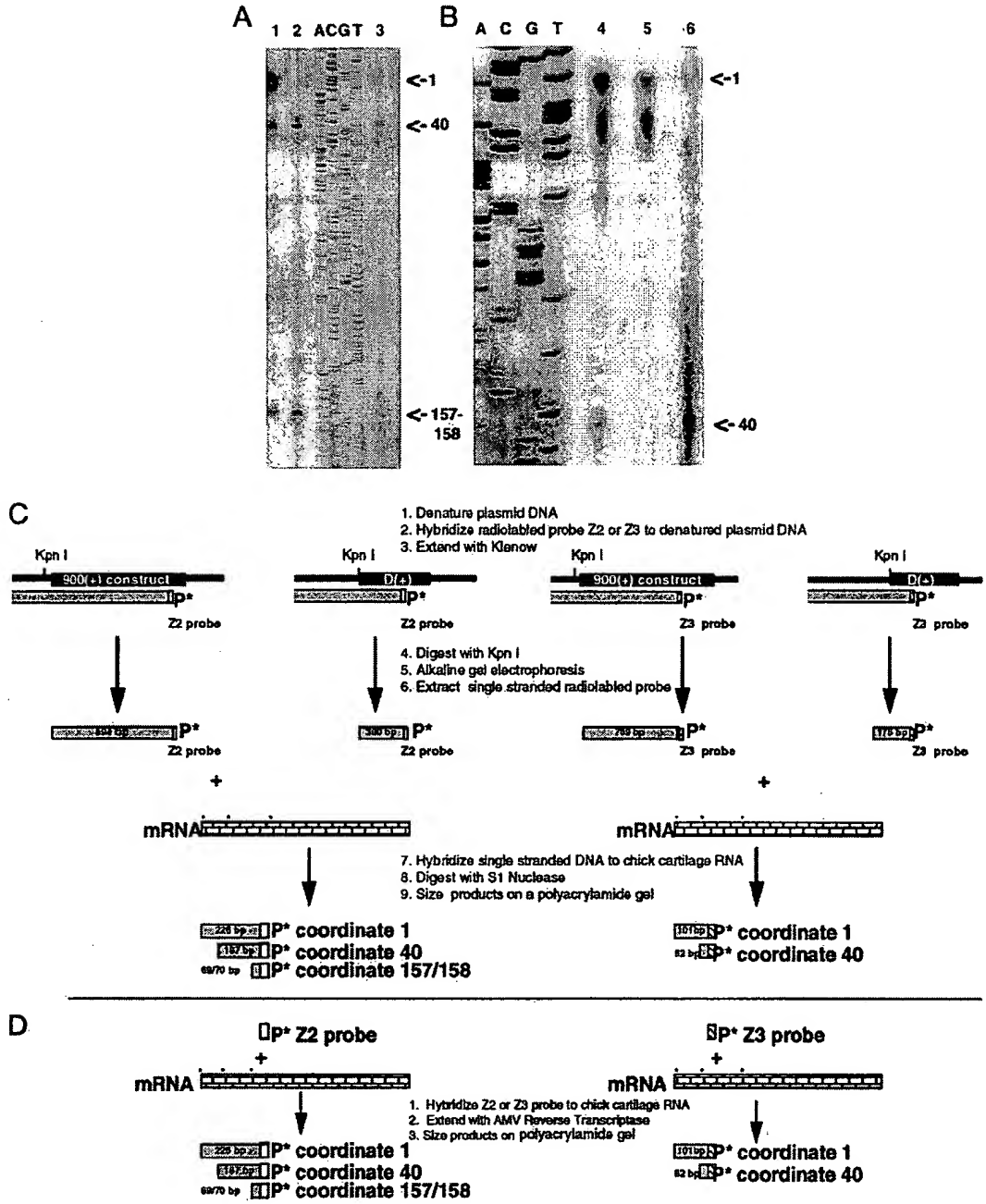

FIG. 4. **S1 analysis and primer extension.** Conditions are described under "Experimental Procedures." *A,* the results of S1 analysis, sequencing, and primer extension from the oligonucleotide Z2. *Lane 1,* S1 protection bands resulting from the D(+)-derived probe spanning nucleotides −69 to +226; *lane 2,* products resulting from the probe derived from the 900(+) construct, spanning the region −638 to +226; *lane 3,* results of a primer extension experiment using ^{32}P-end-labeled oligonucleotide Z2. *B,* results of S1 analysis, sequencing, and primer extension from the oligonucleotide Z3. Conditions for S1 analysis and primer extension were the same as for *A. Lane 4,* S1 protection products from the single-stranded DNA probe spanning the region −69 to +101, derived from D(+); *lane 5,* products from the probe spanning the region −638 to +101, derived from 900(+); *lane 6,* results of a primer extension experiment using ^{32}P-end-labeled oligonucleotide Z3. *Arrows,* the location of the major bands. The bands at position 157–158 consistently appear as a doublet in both S1 analysis and primer extension experiments. Only bands that were generated in both types of experiments were marked; other bands are potentially artifactual because they cannot be duplicated in the complementary experiment. *C* and *D* schematically show the design and results of the S1 protection and primer extension experiments, respectively. *Open boxes,* the radiolabeled oligonucleotide Z2; *slashed boxes,* the radiolabeled oligonucleotide Z3. *Bricks,* RNA; * above the RNA, the determined transcription start sites.

promoted by the construct Ag-1(+). Transfected fibroblasts showed little difference in luciferase activity from Ag-1(+) to 1300(+); the latter was slightly lower. Deletion of another 500 bp from the 5′ end (including a CIIS2 site) generated the construct 900(+); this deletion had a dramatic effect, because both chondrocyte and fibroblast luciferase yields nearly tripled when compared with assays of the original Ag-1(+) construct (to 140- and 30-fold, respectively). Although chondrocyte activity remained substantially higher than that in fibroblasts, there was a greater proportional increase in luciferase activity in fibroblasts, 260% when compared with the 1300(+) construct in fibroblasts *versus* a 160% increase in chondrocytes. This increase may be due to loss of tissue specificity or to coincidental but independent effects of silencers in both cell types.

Removal of approximately 400 additional bp from the upstream end of the 900(+) construct (including another CIIS2

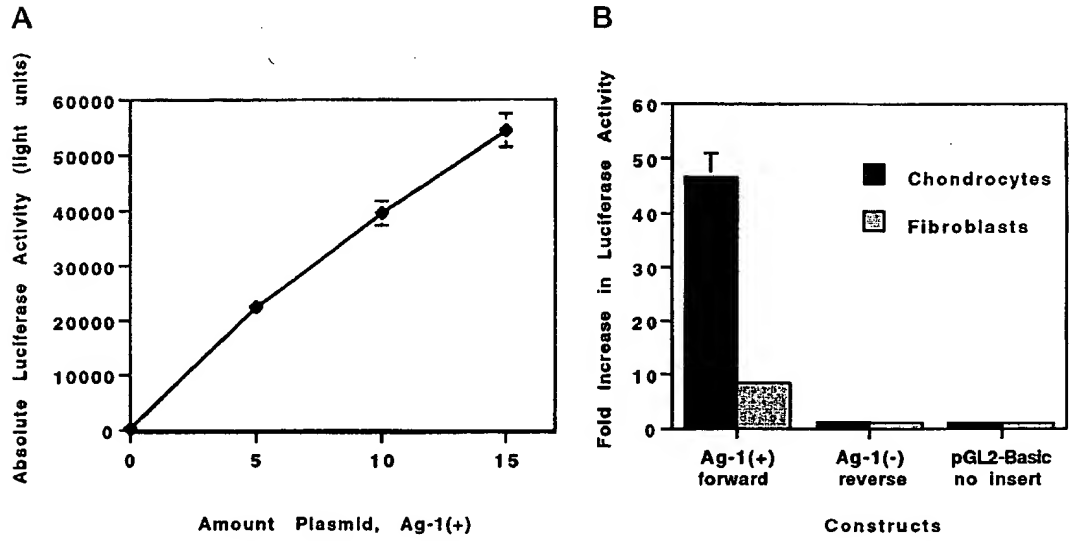

FIG. 5. **Promoter activity in the 5′ flanking region of the chick aggrecan gene.** *A*, the dose-dependent luciferase activity curve resulting from the expression of the Ag-1(+) construct. Ag-1(+) is the 1.8-kb promoter/enhancer region from the aggrecan gene placed in the reporter vector pGL2-Basic. Various amounts of plasmid, ranging from 5 to 15 pmol, were transfected into day-14 chick embryo sternal chondrocytes. Duplicate plates were transfected, and each plate was assayed for luciferase activity three times. An average value and S.D. (*bars*) were determined for all six assays at each data point. Results were normalized by cotransfection of 5 μg of a β-galactosidase reporter plasmid (Promega). *B*, the orientation and cell type specificity of the construct Ag-1(+). The activity of the reporter vector, pGL2-Basic, with no insert was defined as one and used to calculate the relative activities of the other constructs. Again, β-galactosidase expression was used to normalize the plates for transfection efficiency and cell loss; statistical analysis was done in the same fashion as above. Both day-14 chick sternal chondrocytes and day-10 chick fibroblasts were transfected. At the time of transfection, cell density per dish was approximately 5 million. The transfection was allowed to proceed for 36 h.

site) produced the 500(+) construct. Promoter activity in chondrocytes returned to approximately 50-fold, similar to that assayed for the constructs Ag-1(+) and 1300(+); yet in fibroblasts, luciferase activity of the 500(+) construct was only slightly lower than that seen for the 900(+) construct (Fig. 6). This finding suggests that the upstream half of 900(+) may contain enhancer elements that are used in chondrocytes.

A newly generated construct A(+), 590 bp, was made that was similar to the 500(+) construct, except that the insert contained the 3′ stretch of poly(dA) regions and 36 bp in the 5′ direction to include the putative IgHC.21 site (Fig. 3). These changes produced a modest increase in luciferase activity in chondrocytes only. Measured luciferase activity in fibroblasts modestly decreased when compared with the luciferase activity measured from fibroblasts transfected with the 500(+) construct. The deletion construct B(+), 547 bp, which does not contain the IgHC.21 site, lost approximately 40% of the activity of the A(+) construct in chondrocytes; the activity in fibroblasts was reduced by 70%, resulting in luciferase activity as low as that seen for many of the (−) constructs. A further deletion construct, D(+), 376 bp, which included only the three transcription start sites and the putative Sp1 and AP2 binding sites, produced a significant amount of luciferase activity in chondrocytes (nearly 60-fold), and in transfected fibroblasts luciferase activity was equivalent to the 1.8-kb Ag-1(+) construct. The D(+) construct deleted the poly(dT) region but included the poly(dA) region. The 308-bp construct, E(+), included the three major start sites at positions 1, 40, and 157/158 but did not include the consensus sequences Sp1-CS4, GR-MT-IIA, and AP-2-CS4. Deletion of these potential nuclear factor binding sites caused a 75% loss of activity in chondrocytes while not substantially altering luciferase activity in transfected fibroblasts. Construct E(+) had comparable luciferase activity in both chondrocytes and fibroblasts of approximately 15-fold when compared with the no-insert control vector. The 140-bp construct F(+) did not include any of the determined starting sites and produced modest luciferase activity in transfected chondrocytes and baseline luciferase activity in transfected fibroblasts. In all but one instance, the reverse orientation constructs of all of these genomic fragments yielded minimal luciferase activity in both transfected chondrocytes and fibroblasts. That exception, the activities seen for the 500(−) construct, suggests that some low-level promoter activities may result from largely accidental sequence assemblages. In sum, the data suggest the following functional roles for portions of the aggrecan 5′ flanking sequence in the two cell types: 1) general repression upstream of the pr900 site, especially between −638 and −1038 (pr1300); 2) strong chondrocyte-specific enhancement in the pr900-pr500 interval (−638 to −247); 3) a positive element, possibly IgHC.21, occurs in the small prA-prB interval (−283 to −240); 4) the prB-prD segment (−240 to −69) has a negative role, strongest in fibroblasts; and 5) the small (−69 to −1) pD-prE interval, bearing SP1 and AP-2 elements, is stimulatory in chondrocytes. It is also apparent that constructs lacking either the dT or dA tracts (*e.g.* 900(+) and D(+)) are quite active; therefore, interaction between these repeats is not required for promoter function in this system.

DISCUSSION

We have found that a 1.8-kb genomic fragment from the 5′ end of the chick aggrecan gene is able to drive expression of the pGL2-Basic luciferase reporter gene in a tissue-specific manner. Determining the sequence of this construct revealed more than 202 potential transcription factor binding sites. This structural information allowed us to proceed with a functional analysis of the effects of potentially active *cis* elements that may confer tissue and developmental specificity on expression of the aggrecan gene by using a series of nested deletion constructs. These sequences ranged from the full 1.8 kb (Ag-1(+)) to a minimal 140-bp construct (F+).

Of the numerous potential *cis* elements found in the Ag-1 sequence, several are of particular interest with respect to control of aggrecan expression. Positions −873 and −721 in the Ag-1 sequence are the 5′ ends of two copies of the sequence CACCTCC (CIIS2), which has been suggested to be a silencer

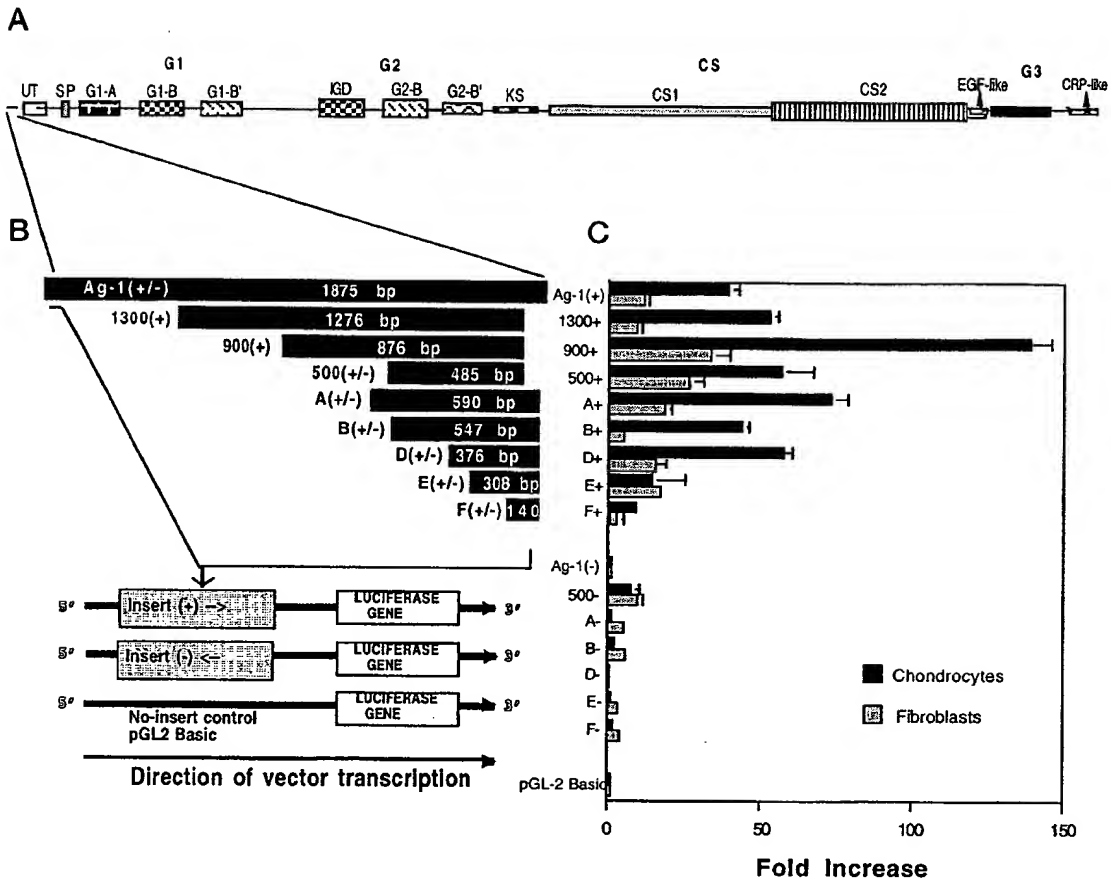

FIG. 6. **Structure and differential promoter functions of the aggrecan 5′ flanking region.** *A*, a schematic of the genomic structure of the chick S103L-reactive CSPG (aggrecan) gene. *B*, the set of deletion constructs. Inserts derived from the aggrecan promoter/enhancer region were ligated into the pGL2-Basic (Promega) reporter vector, which carries the luciferase gene. Both the forward and reverse orientations were constructed as indicated by (+) or (−). Subsequent deletion constructs were generated by PCR using the construct Ag-1(+) as a template (see "Experimental Procedures." *C*, relative luciferase promoter activity of the various deletion constructs. The activity of the reporter vector with no insert was defined as one and used to calculate the activities of the other constructs. Duplicate plates were transfected, and each plate was assayed for luciferase activity three times. All experimental details are as presented in the legend to Fig. 5.

motif in the *COL2A1* promoter (29). This particular sequence has been shown to inhibit transcription of the type II collagen promoter in fibroblasts while not significantly changing expression in chondrocytes (29). Indeed, this seems to be consistent with our results because deletion of these two motifs from the 1300(+) to 900(+) constructs reduced the cell type specificity of luciferase expression while the overall promoter activities increased. This motif is also present in the promoter region of *COL4A2*; however, tissue-specific regulation in fibroblasts *versus* chondrocytes remains to be investigated in this system (30).

The chick aggrecan 5′ flanking region contains a second silencer consensus sequence, (CIIS1) ACCCTCTCT (29) at position 127, which is also found in *COL2A1*. The CIIS1 sequence occurs in an interspersed rat repetitive sequence (31) and in another repetitive sequence found in the avian genome named the CR1 element (32, 33). Further negative regulatory functions have been shown in the chick lysozyme gene (34), rat insulin gene (31), mouse IgH gene (35), human β-interferon gene (36), and the human ε-globin gene (37). In the Ag-1 sequence, this motif is located within 200 bp downstream of the putative Sp1 site. A "push and pull" mechanism has been proposed for transcriptional regulation in two systems, the low density lipoprotein receptor gene and the *COL2A1* gene (29, 38). This model proposes that the sterol-dependent binding of a protein to a consensus sequence could inhibit the positive activation of a nearby Sp1 binding site (38); such a silencer element acting in a "push and pull" mechanism could likewise be responsible for the temporal and tissue-specific regulation of the aggrecan gene.

The Ag-1 sequence contains one putative NF-I site at position −1282. The NF-I proteins are transcriptional activators derived from a multigene protein family in the vertebrate phylum (39–42). Chick tissues contain NF-I products that are derived from four separate genes that have the potential of producing 12 isoforms (42). Recently, it has been shown that the silencer SI is very similar to the NF-I/CTF family, and an additional silencer, SII, is similar to an NF-I/CTF half site (43) This suggests that NF-I-related proteins can mediate transcriptional repression in cells of mesenchymal origin (42). Our sequence does not contain the sequence motifs of SI or SII, but Szabo *et al.* (43) suggest that the NF-I family of regulator proteins can be modulated as silencers in addition to their previously accepted role as activators. The presence of a putative NF-I site raises the possibility of mesenchyme-specific regulation controlled by this element in addition to possible modulation by unreported silencers, thus creating a more dynamic system than one based solely on NF-I activation.

From footprinting analysis, Long and Linsenmayer (44) reported a novel transcription factor binding sequence, ACACACAGA, acting in the regulation of *COL10A1*, and suggested that this factor may act as a silencer. The proximal promoter region of *COL10A1* is responsible for regulating expression in hypertrophic chondrocytes (44). Our reported sequence contains four positions, −1140, −491, 151, and 214, where the

CACACA motif is present. Perhaps these sequences are involved in chondrocyte-specific expression of aggrecan. The CACACA motif may also be relevant because repeats of $(CA)_n$ are markers for Z-DNA formation, contributing to secondary structure (45). Moreover, this motif has been shown to be a potential hot spot for recombination and can contribute to gene expression (26). Clustering of these sequences near the transcriptional start sites that have been identified for chick aggrecan may contribute to the mechanism of transcriptional regulation by altering DNA secondary structure.

The chick aggrecan promoter exhibits <40% sequence similarity to either the mouse promoter (19) or the 120-bp rat (20) promoter fragment, indicating that this promoter/enhancer region is not highly conserved across the taxa. Interestingly, the untranslated first exon in chick aggrecan contains less than 45% similarity compared with rat, mouse, or human sequences (19, 20, 46). Although the lack of identifiable similarity between the chick and mammalian aggrecan first exons might be attributable to the existence of fewer selection pressures on an untranslated sequence, this argument is not readily extended to promoter sequences. Also puzzling is that although the rat and mouse promoter sequences share 93% identity with each other, none of the described transcriptional start sites coincide with each other in these two similar promoter regions.

There are, however, similarities in TATA-binding motifs among promoters of cartilage-specific genes. As is the case for the mouse and rat aggrecan promoter regions, the chick 5′ flanking sequence lacks a classical TATA box and contains multiple transcriptional start sites (19, 20). Although a TATA-less promoter with multiple GC-rich regions is the hallmark of many housekeeping genes (47), many other genes that are temporally regulated have been shown to have promoters with similar structures (48, 49). It is interesting that the 5′ flanking sequence of the chick link protein gene also contains multiple transcription start sites and lacks a classical TATA box (50); rather, it has a TATA motif-like sequence TCTAA (51). The chick aggrecan sequence contains two TCTAA motifs, one that is 31 bp and another that is 94 bp upstream of the start sites at positions 40 and 157–158, respectively (Fig. 3). The TCTAA sequence is also present in the human and chick link protein promoter region (50, 52) and in the serine/glycine-rich proteoglycan (51). However, human link protein has only one transcription start site (52). Thus, it would be interesting to determine whether the human aggrecan sequence has only one transcription start site, which would provide further evidence for similarity in the evolution of the link protein and the aggrecan genes, as has been suggested (13).

Overall, this study has established the 5′ flanking sequence as having three major transcription start sites in addition to several putative *cis* elements and a potential secondary structure that may control expression of the aggrecan gene. We have demonstrated tissue-specific promoter activity with the 1.8-kb region and have systematically mapped subregions that produce activation or repression of downstream reporter genes in two cell types in culture. This study paves the way for more directed studies of the individual *cis* elements identified and their interaction with *trans*-acting factors so that we may better understand the mechanisms by which the aggrecan gene is regulated.

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A new long form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene

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Transcripts for a new form of Sox5, called L-Sox5, and Sox6 are coexpressed with *Sox9* in all chondrogenic sites of mouse embryos. A coiled-coil domain located in the N-terminal part of L-Sox5, and absent in Sox5, showed >90% identity with a similar domain in Sox6 and mediated homodimerization and heterodimerization with Sox6. Dimerization of L-Sox5/Sox6 greatly increased efficiency of binding of the two Sox proteins to DNA containing adjacent HMG sites. L-Sox5, Sox6 and Sox9 cooperatively activated expression of the chondrocyte differentiation marker *Col2a1* in 10T1/2 and MC615 cells. A 48 bp chondrocyte-specific enhancer in this gene, which contains several HMG-like sites that are necessary for enhancer activity, bound the three Sox proteins and was cooperatively activated by the three Sox proteins in non-chondrogenic cells. Our data suggest that L-Sox5/Sox6 and Sox9, which belong to two different classes of Sox transcription factors, cooperate with each other in expression of *Col2a1* and possibly other genes of the chondrocytic program.

Keywords: chondrogenesis/collagen2/Sox5/Sox6/Sox9

Introduction

Sox (Sry-type HMG box) proteins, which form a subfamily of DNA-binding proteins with a high-mobility-group (HMG) domain, have critical functions in a number of developmental processes, including sex determination, neurogenesis and skeleton formation (Laudet *et al.*, 1993; Pevny and Lovell-Badge, 1997; Southard-Smith *et al.*, 1998). Individual members of the Sox family show >50% identity in their HMG domain to Sry, the testis-determining factor (Wright *et al.*, 1993). An essential role for SOX9 in skeleton formation was demonstrated with the identification of mutations in *SOX9* in patients with campomelic dysplasia (Foster *et al.*, 1994; Wagner *et al.*, 1994; Kwok *et al.*, 1995; Meyer *et al.*, 1997). This disease is characterized by severe malformations of essentially all cartilage-derived structures and is also often associated with XY sex reversal (Houston *et al.*, 1983; Mansour *et al.*, 1995).

Cartilage formation is a complex and essential process in vertebrates. Cartilages are obligatory templates for the formation of endochondral bones during development and also constitute permanent skeletal structures in the respiratory tract, in articular joints and other organs. Chondrocytes differentiate following condensation of mesenchymal cells in different locations of the embryo, including the frontonasal mass, branchial arches, sclerotomes and limb buds. Typically, chondrocytes express a set of genes encoding cartilage-specific extracellular matrix components such as collagen II (encoded by the *Col2a1* gene), collagens IX and XI, and aggrecan. In growth plates, chondrocytes undergo further differentiation and hypertrophy, producing a matrix in which type X collagen is abundant and calcification occurs. Apoptosis follows and cartilage is replaced by bone. Our understanding of chondrogenesis at the molecular level is still limited. Several cytokines, including bone morphogenetic proteins, Indian Hedgehog, parathyroid hormone-related peptide and fibroblast growth factors, are involved in either skeletal patterning or discrete steps of the chondrogenic pathway, and several transcription factors, such as Hox and Pax family members, are involved in patterning of skeletal primordia (Cancedda *et al.*, 1995; Erlebacher *et al.*, 1995; Hall and Miyake, 1995). However, less is known about the transcription factors that control the determinative switch for chondrocyte differentiation and the activation of marker genes at each step of the chondrogenic cascade.

Our laboratory has used genes for specific cartilage matrix components to identify transcription factors that control gene expression in chondrocytes. We have shown that a multimerized 48 bp sequence in the first intron of *Col2a1* is sufficient to confer chondrocyte-specific expression both in transgenic mice and in transient transfection of cultured cells (Lefebvre *et al.*, 1996). SOX9 binds to the enhancer and activates *Col2a1* constructs in transient transfections of non-chondrocytic cells (Lefebvre *et al.*, 1997) and in transgenic mice (Bell *et al.*, 1997). SOX9 also activates the *Col2a1* gene when ectopically expressed in some non-cartilaginous sites in transgenic mice (Bell *et al.*, 1997). Moreover, *Sox9* is expressed along with *Col2a1* during chondrogenesis in mouse embryos (Wright *et al.*, 1995; Ng *et al.*, 1997; Zhao *et al.*, 1997). Therefore, direct activation of *COL2A1* is believed to be an important function of SOX9 in chondrogenesis (Lefebvre and de Crombrugghe, 1998).

Several lines of evidence suggest that other transcription factors in addition to SOX9 may be needed to specify the high-level expression of *COL2A1* in chondrocytes. *Sox9* is expressed in cells that do not express *Col2a1*, such as those in genital ridges and specific areas of the embryonic heart (Ng *et al.*, 1997; Zhao *et al.*, 1997). Sox9 is highly expressed in the Sertoli cells of the testis and is involved in male gonad differentiation (Kent *et al.*, 1996; Morais da Silva *et al.*, 1996). The target genes of Sox9 in these

cells and in chondrocytes are not clearly defined, but the phenotypes of these two cell types are so different that it is likely that Sox9 contributes to their differentiation by controlling expression of different genes. Different functions of Sox9 in these cells must be specified by differential expression of other factors. Ectopic expression of *SOX9* in transfected cells, in which *Col2a1* was silent, did not result in *Col2a1* activation (V.Lefebvre and B.de Crombrugghe, unpublished data), and ectopic expression of *SOX9* in transgenic mice led to activation of *Col2a1* only in a subset of tissues within the domain of ectopic expression of *SOX9* (Bell *et al.*, 1997). We therefore hypothesized that other factors either activate or derepress *SOX9* or cooperate with *SOX9* in *COL2A1*-expressing cells.

We recently reported that the 48 bp enhancer of *Col2a1* formed a large and abundant complex with nuclear proteins from chondrocytes but not from other cells (Zhou *et al.*, 1998). These proteins were designated CSEPs, for chondrocyte-specific enhancer-binding proteins. They included Sox9 and unidentified protein(s). CSEPs appeared to contact the 48 bp DNA at several sites homologous to a consensus for HMG-domain proteins. Mutagenesis demonstrated a good correlation between binding of CSEPs to DNA and enhancer activity in chondrocytes, both in transient transfection experiments and in transgenic mice. In addition, we showed that two chondrocyte-specific enhancer elements located in the *Col11a2* promoter contained HMG-like sites that were essential for enhancer activity and formation of an enhancer–CSEP-like complex (Bridgewater *et al.*, 1998). These results suggested that other HMG-domain proteins cooperate with Sox9 to generate *Col2a1* and *Col11a2* enhancer activity and presumably gene expression in chondrocytes.

We show here that in addition to Sox9, CSEPs are composed of a new long form of Sox5 (L-Sox5), and of Sox6, which both are members of a Sox subclass different from that of Sox9. L-Sox5 and Sox6 harbor a coiled-coil domain that mediates protein dimerization and efficient binding to adjacent HMG DNA sites. The three *Sox* genes are coexpressed in chondrogenesis and cooperate in *Col2a1* activation. Our data strongly suggest that L-Sox5, Sox6 and Sox9 together contribute to control *Col2a1*, and perhaps other important genes of the chondrocyte phenotype.

Results

A long form of Sox5 (L-Sox5), Sox6 and Sox9 form complexes with the 48-bp Col2a1 enhancer

The CSEP proteins that form a chondrocyte-specific complex with the 48 bp *Col2a1* enhancer were previously shown to include a protein or proteins with an apparent M_r of 75–95 kDa (Zhou *et al.*, 1998). These proteins exhibited DNA-binding properties of HMG-domain proteins, including binding to several HMG-like sites in the *Col2a1* 48 bp enhancer, binding to a probe containing a consensus binding site for HMG-domain proteins (1HMG probe), binding to the minor groove of DNA and binding to DNA in the presence of poly(dG–dC) but not poly(dI–dC) (Zhou *et al.*, 1998). We also obtained evidence that CSEP bound with high affinity to a tandem dimer of the 1HMG probe (2HMG probe) both in EMSA and in Southwestern blots (data not shown).

On the basis of these results, the 2HMG probe was chosen to clone cDNAs for CSEPs by the Southwestern screening approach. cDNA expression libraries were made from primary chondrocytes of newborn mouse ribs. Several clones that showed stronger binding to the 2HMG probe in the presence of poly(dG–dC) than poly(dI–dC) encoded sequences of Sox5 or Sox6. Interestingly, whereas the previously reported transcript for Sox5 had a length of 2 kb and encoded a 43 kDa protein (Denny *et al.*, 1992), the *Sox5* cDNA that was reconstituted from overlapping clones was 3.9 kb long and encoded a 75 kDa protein corresponding to Sox5 with an additional N-terminal sequence (data submitted to DDBJ/EMBL/GenBank; see later, in Figure 4). This long form of Sox5 was designated L-Sox5. Sox6 cDNA clones encoded Sox6 isoforms identical or very similar to those described for testis (data submitted to DDBJ/EMBL/GenBank; see later, in Figure 4).

Antibodies were generated against the C-termini of Sox5, Sox6 and Sox9, and affinity purified. In Western blotting, each antibody species specifically recognized its Sox protein target in extracts of fibroblasts transfected with Sox expression plasmids (Figure 1A). In EMSA, each antibody preparation specifically supershifted complexes formed between DNA and its target (Figure 1B). In Western blots of RCS cell extracts, the antibodies strongly reacted with proteins at the level of L-Sox5, Sox6 and Sox9 (Figure 1C), indicating that each of these Sox proteins was indeed made by these cells. The same reactions were seen with extracts of primary chondrocytes and chondrocytic MC615 cells (data not shown), but not with extracts of BALB/3T3 (Figure 1C) or 10T1/2 fibroblasts (see control lanes in Figure 1B). Note that Sox5 antibodies showed no reaction in chondrocyte samples at the level of the 43-kDa short form of Sox5. In extracts of adult mouse testis, Sox5 antibodies recognized Sox5 but not L-Sox5 (Denny *et al.*, 1992). It appeared, therefore, that only L-Sox5 is made in chondrocytes, not Sox5, whereas only Sox5 is made in the testis. These protein data are consistent with RNA data, which show that chondrocytes express only the transcript for L-Sox5 and testis only the transcript for short Sox5 (see Figure 2A). In EMSA, L-Sox5 and Sox6 made in transfected fibroblasts formed complexes with the 48 bp *Col2a1* enhancer probe that migrated at the level of the CSEP–DNA complex (Figure 1D). Sox5 did not bind to the 48 bp element. As shown previously (Zhou *et al.*, 1998), SOX9 formed two complexes with the enhancer, a major one migrating faster than the CSEP–DNA complex and a minor one migrating at the level of the CSEP–DNA complex (Figure 1D). Sox5, Sox6 and Sox9 antibodies were each able to partially supershift the CSEP–48-bp-*Col2a1* enhancer complex formed with RCS cell nuclear extracts (Figure 1E), indicating that each of these Sox proteins contributed to complex formation. A virtually complete supershift of the CSEP–DNA complex was obtained by incubating EMSA reactions with both Sox5 and Sox6 antibodies. No further supershift was visible when the three Sox protein antibodies were included in the reactions. The same results were obtained with primary chondrocyte extracts (data

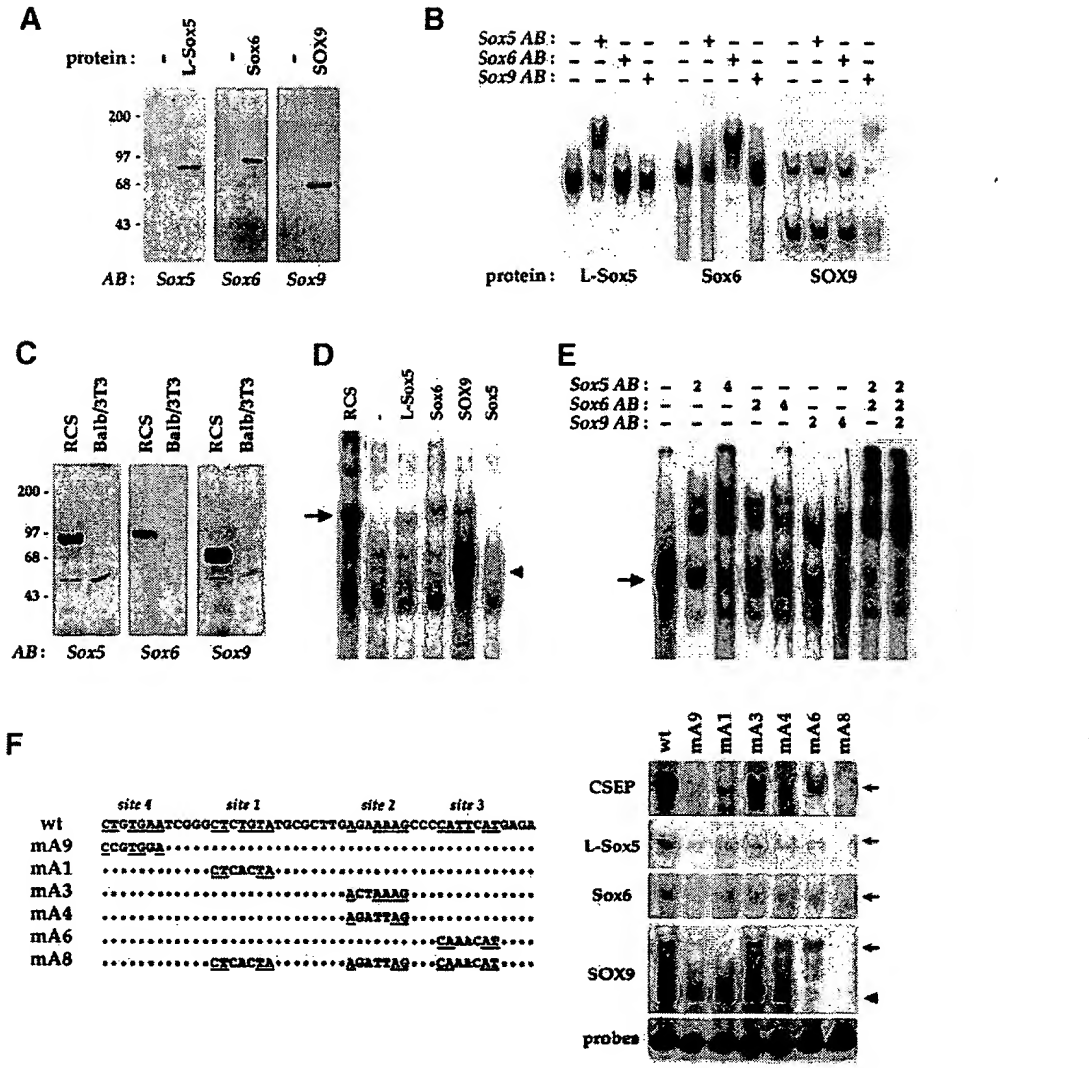


Fig. 1. L-Sox5, Sox6 and Sox9 are present in chondrocytes and form the CSEP–*Col2a1*-48-bp enhancer complex. **(A)** Antibodies (AB) against Sox5, Sox6 and SOX9 specifically recognized their target in Western blots. Samples were extracts of 10T1/2 fibroblasts transfected with expression plasmids encoding no protein (–), L-Sox5, Sox6A or SOX9. The M_r of protein standards ($\times 10^3$) is indicated. Reaction with antibodies is seen at the level expected for each Sox protein. **(B)** Antibodies against Sox5, Sox6 and Sox9 specifically supershifted the complex of their target with DNA. L-Sox5 and Sox6 protein samples were cell extracts from 10T1/2 fibroblasts transfected with Sox expression plasmids. The SOX9 sample was the product of *in vitro* transcription/translation with SOX9 plasmid. Samples were used in EMSA with the 2HMG probe. Two microliters of SOX crude antiserums were included, as indicated. A preimmune serum was used as a control (first lane for each protein). None of the complexes seen in the figure was seen in samples containing no SOX protein (data not shown). SOX9 synthesized *in vitro* formed two distinct complexes with the 2HMG probe. The lower and upper complexes probably involved one and two molecules of SOX9 per molecule of DNA, respectively. **(C)** Antibodies against Sox5, Sox6 and Sox9 identified their target in Western blots of nuclear extracts of RCS cells but not BALB/3T3 fibroblasts. An intense reaction with antibodies was seen at the level of each Sox protein. **(D)** L-Sox5, Sox6 and SOX9 bound to the 48 bp probe. 10T1/2 fibroblasts were transfected with expression plasmids for no protein (–), L-Sox5, Sox6, SOX9 or Sox5. Extracts of these cells and nuclear extracts of RCS cells were used in EMSA with the 48 bp *Col2a1* probe. L-Sox5 and Sox6 formed a complex with the probe that ran with a mobility similar to that of the CSEP–DNA complex. As shown previously (Zhou *et al.*, 1998), SOX9 formed two complexes with the *Col2a1* enhancer probe, a minor one that migrated at the level of the CSEP–DNA complex (arrow), and a major one that migrated faster (arrowhead). **(E)** Antibodies against Sox5, Sox6 and Sox9 supershifted the CSEP–48-bp-*Col2a1* complex. Two or four microliters of SOX antiserums (AB) were included in EMSA of RCS nuclear extracts with the 48 bp *Col2a1* probe, as indicated. Sox5 preimmune serum was used in the control reaction (first lane). None of the preimmune serums supershifted any protein–DNA complex (data not shown). As described previously (Zhou *et al.*, 1998), Sox9 present in RCS extracts did not form a fast migrating complex with DNA (as seen in D). **(F)** EMSA with wild-type and mutant 48 bp *Col2a1* probes. The upper strand of the wild-type 48 bp element (wt) is shown from 5′ to 3′. Sites 1–4 are 7 bp HMG-like binding sites. Nucleotides corresponding to those of the heptamer consensus HMG site C[A/T]TTG[A/T][A/T] are underlined. Mutated sites are spelt out, whereas wild-type nucleotides are indicated by dots. Sites 1–3 and mutants mA1, mA3, mA4, mA6 and mA8 were described previously (Zhou *et al.*, 1998). Note that site 2 in mA3 harbors the same five consensus nucleotides as wild-type site 2, whereas, in other mutations, sites 1–4 retained only three or four consensus nucleotides. Binding of CSEP to wild-type and mutant 48 bp probes was tested using RCS nuclear extracts. Binding of L-Sox5, Sox6 and SOX9 was tested using extracts of 10T1/2 cells transfected with either one of the Sox protein expression plasmids. All probes had the same radioactivity. Arrow, migration level of CSEP. Arrowhead, fast-migrating complex of SOX9 with DNA.

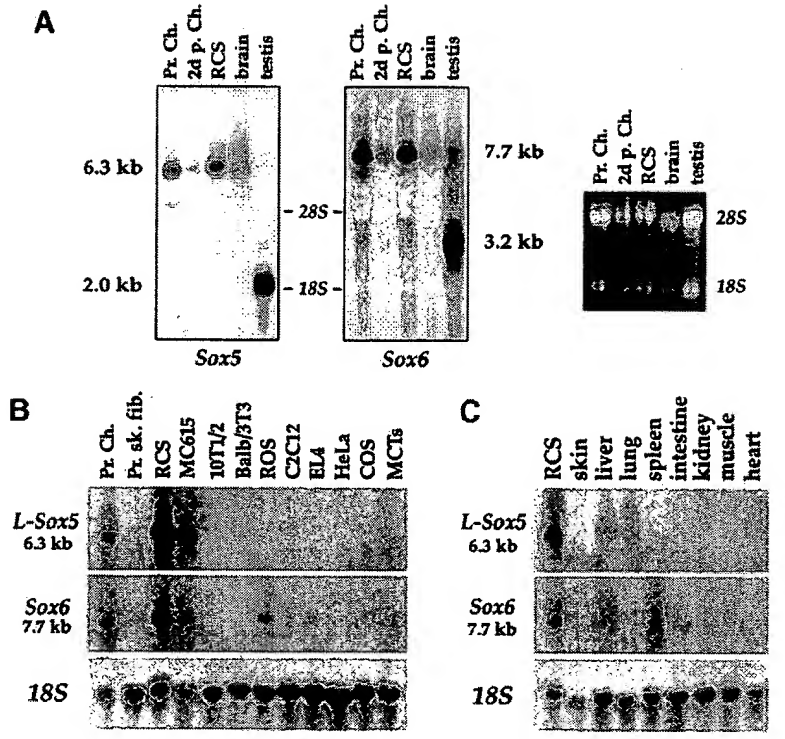


Fig. 2. Long transcripts of *Sox6* and *Sox5* are expressed at high levels in chondrocytes. (**A**) Northern blot with total RNA. Samples were as follows: Pr. Ch., primary chondrocytes; 2d p. Ch., chondrocytes at the second passage in culture; RCS cells; newborn mouse brain and adult mouse testis. Hybridization was performed with probes that recognized both the long and short transcripts of either *Sox5* or *Sox6*. Staining of 28S and 18S rRNA is shown as the reference for RNA loading. The sizes of *Sox5* and *Sox6* transcripts were calculated by comparison with the migration of RNA standards. (**B**) Northern blot with total RNA. Samples were as follows: Pr. Ch., primary chondrocytes; Pr. sk. fibr., primary skin fibroblasts from newborn mice; RCS cells; MC615 mouse immortalized chondrocytic cells at an early passage; 10T1/2 and BALB/3T3 mouse embryo fibroblasts; ROS rat osteosarcoma cells; C_2C_{12} mouse myoblastic cells; EL4 mouse lymphoma cells; HeLa human carcinoma cells; COS monkey kidney cells; and MCTs, mouse immortalized chondrocytes. Hybridization was performed with probes for the long and short transcripts of either *Sox5* or *Sox6*. Only signals at the level of the long transcripts are shown. No significant hybridization was seen at the level of the short transcripts in any sample (data not shown). Hybridization with an 18S rRNA probe is shown as the control for RNA loading. RNA samples were the same as in Lefebvre *et al.* (1997). (**C**) Same Northern blot as in (B), but with total RNA from newborn mouse tissues.

not shown). L-Sox5 and Sox6 therefore appeared to be predominant CSEP components.

Altogether these results indicated that three different Sox proteins, L-Sox5, Sox6 and Sox9, were present in chondrocytes and bound to the 48 bp *Col2a1* element. L-Sox5 is a long product of the *Sox5* gene that has not been identified previously.

L-Sox5, Sox6 and Sox9 contact several HMG-like sites in the 48 bp Col2a1 enhancer

The *Col2a1* 48 bp enhancer contains four HMG-like sites, each containing 5 or 6 bp of the 7 bp consensus HMG site (Figure 1F). Previously we showed that mutations that disrupted any one of sites 1–3 inhibited enhancer activity in chondrocytes in transgenic mice and partially inhibited binding of CSEP to the enhancer (mutants mA1, mA4 and mA6 in Figure 1F; Zhou *et al.*, 1998). A mutation that disrupted site 4 also impaired binding of CSEP to the enhancer (mutant mA9 in Figure 1F). The effect of this mutation on enhancer activity has not been tested. A mutation that preserved all five HMG consensus nucleotides of site 2 did not affect the binding of CSEP (mA3 in Figure 1F) and only weakly inhibited enhancer activity in RCS cells (Zhou *et al.*, 1998). A mutation that disrupted three sites completely inhibited binding of CSEP (mA8 in Figure 1F; Zhou *et al.*, 1998). We also reported previously that subfragments of the 48 bp element containing either one of the four HMG-like sites alone were unable to bind CSEP or to compete with the 48 bp element for binding of CSEP (Lefebvre *et al.*, 1996, 1997; Zhou *et al.*, 1998; our data, not shown). These results indicated that the four sites of the 48 bp element cooperatively contributed to formation of the CSEP–enhancer complex.

As described previously (Lefebvre *et al.*, 1997; Zhou *et al.*, 1998), the faster-migrating SOX9–DNA complex was inhibited by mutation of site 3 but not by mutation of other sites (Figure 1F). In contrast, the slower-migrating SOX9–DNA complex was partially inhibited by mutations disrupting any of the four sites and was completely inhibited by mutation of several sites. These results are consistent with the notion that the faster-migrating complex was formed by binding of one molecule of SOX9 to site 3, whereas the slower-migrating complex was formed by cooperative binding of two or more SOX9 molecules to several sites on each DNA molecule.

Consistent with the results obtained with CSEP, binding of L-Sox5 or Sox6 to the 48 bp enhancer was partially inhibited by mutations that disrupted any one of the four HMG-like sites and completely inhibited by a mutation of several sites (Figure 1F). Mutation mA1 slightly but reproducibly inhibited binding of L-Sox5 and Sox6 to DNA. As seen with CSEP, L-Sox5 and Sox6 were unable

to bind to DNA probes harboring only one of the *Col2a1* HMG-like sites (data not shown). Taken together, these data indicated that L-Sox5 and Sox6 were able to contact cooperatively all four HMG-like sites of the *Col2a1* 48 bp enhancer.

In chondrocyte nuclear extracts, the faster-migrating complex of Sox9 with the 48 bp enhancer was not seen (Zhou *et al.*, 1998; compare also extracts of RCS cells and extracts of SOX9-transfected 10T1/2 cells in Figure 1D), but antibody supershift experiments indicated that Sox9 was present in the slower migrating CSEP complex. This result strongly suggested that, in chondrocytes, Sox9 bound the 48 bp element not as a single molecule but in cooperativity with other Sox9, L-Sox5 or Sox6 molecules.

These data suggest a model whereby the four HMG-like sites of the 48 bp element and the three Sox proteins could participate *in vivo* in the formation of a large protein–enhancer complex that would activate expression of *Col2a1* in chondrocytes. Consistent with this model in which mutation of any of the HMG-like sites would dismantle the protein–enhancer complex, we have observed that disruption of any of sites 1, 2 or 3 resulted in abolition of the activity of the enhancer in chondrocytes of transgenic mice (Zhou *et al.*, 1998).

A search for other HMG consensus and HMG-like sites (with six nucleotides of the heptamer consensus) in 1 kb of *Col2a1* promoter sequence and in a 468 bp element of the first intron (+1878/+2345), which acts as a strong chondrocyte-specific enhancer in transgenic mice (Zhou *et al.*, 1995), revealed the presence of a few scattered sites, but no cluster of two or more binding sites for Sox9, L-Sox5 and Sox6 was found that resembled that of the 48 bp element (data not shown).

Long transcripts of the Sox6 and Sox5 genes are expressed in chondrocytes

Sox5 and *Sox6* were previously shown to be highly expressed in the testis of adult mice (Denny *et al.*, 1992; Connor *et al.*, 1995; Takamatsu *et al.*, 1995). The transcripts were ~2.0 and ~3.2 kb long, respectively. Traces of longer transcripts (~10 kb) were described for *Sox6* in immature mouse testis (Takamatsu *et al.*, 1995) and several tissues of adult mice (Connor *et al.*, 1995), and also for *SOX5* in human fetal brain (Wunderle *et al.*, 1996).

In Northern blots of total RNA, the short transcripts of *Sox6* (3.2 kb) and *Sox5* (2 kb) were abundant in adult mouse testis, as expected (Figure 2A). These short transcripts were not expressed (or were expressed at a very low level in the case of *Sox6*) in chondrocytes or in any cell line or newborn mouse tissue examined (Figure 2A; data not shown). A 6.3 kb *Sox5* transcript and a 7.7 kb *Sox6* transcript were found in similar relative abundance in primary chondrocytes from ribs of newborn mice, as well as in RCS and early-passage MC615 cells (Figure 2A and B). These three chondrocyte cells were previously shown to be well differentiated, expressing *Col2a1* at a high level in parallel with *Sox9* (Lefebvre *et al.*, 1997). When rib chondrocytes were allowed to dedifferentiate by two passages in monolayer culture, *Sox5* and *Sox6* expression sharply declined (Figure 2A), as did *Col2a1* and *Sox9* expression (Lefebvre *et al.*, 1997). Transcripts of *Sox5*, *Sox6* and *Sox9* were found in some non-chondrocytic cell types, but in contrast to chondrocytes, none of the non-chondrocytic cells coexpressed the three *Sox* genes or expressed *Col2a1* at high levels (Figure 2B; Lefebvre *et al.*, 1997).

The long transcripts of *Sox5* and *Sox6* were present in the brain and some other non-cartilaginous tissues of newborn mice (Figure 2A and C). However, no non-cartilaginous tissue, besides brain and testis, was found to coexpress *Sox9*, *Sox5* or and *Sox6* (Figure 2A and C; Lefebvre *et al.*, 1997). In testis, *Sox9* expression is restricted to the somatic Sertoli cells (Kent *et al.*, 1996; Morais da Silva *et al.*, 1996); *Sox5* is expressed as a short transcript in post-meiotic germ cells, mostly in round spermatids (Denny *et al.*, 1992); and, since expression of *Sox6* correlates with that of the protamine gene (Takamatsu *et al.*, 1995), a marker of spermatid differentiation, it is possible that *Sox6* is expressed in testis in the same cells and at the same time as *Sox5*. The three Sox proteins, therefore, do not appear to be expressed in the same cells in testis.

In conclusion, chondrocytes, and perhaps some brain cells, appear to express together long transcripts for *Sox5* and *Sox6* and transcripts for *Sox9*. Expression of these transcripts correlates with expression of *Col2a1* in chondrocytes.

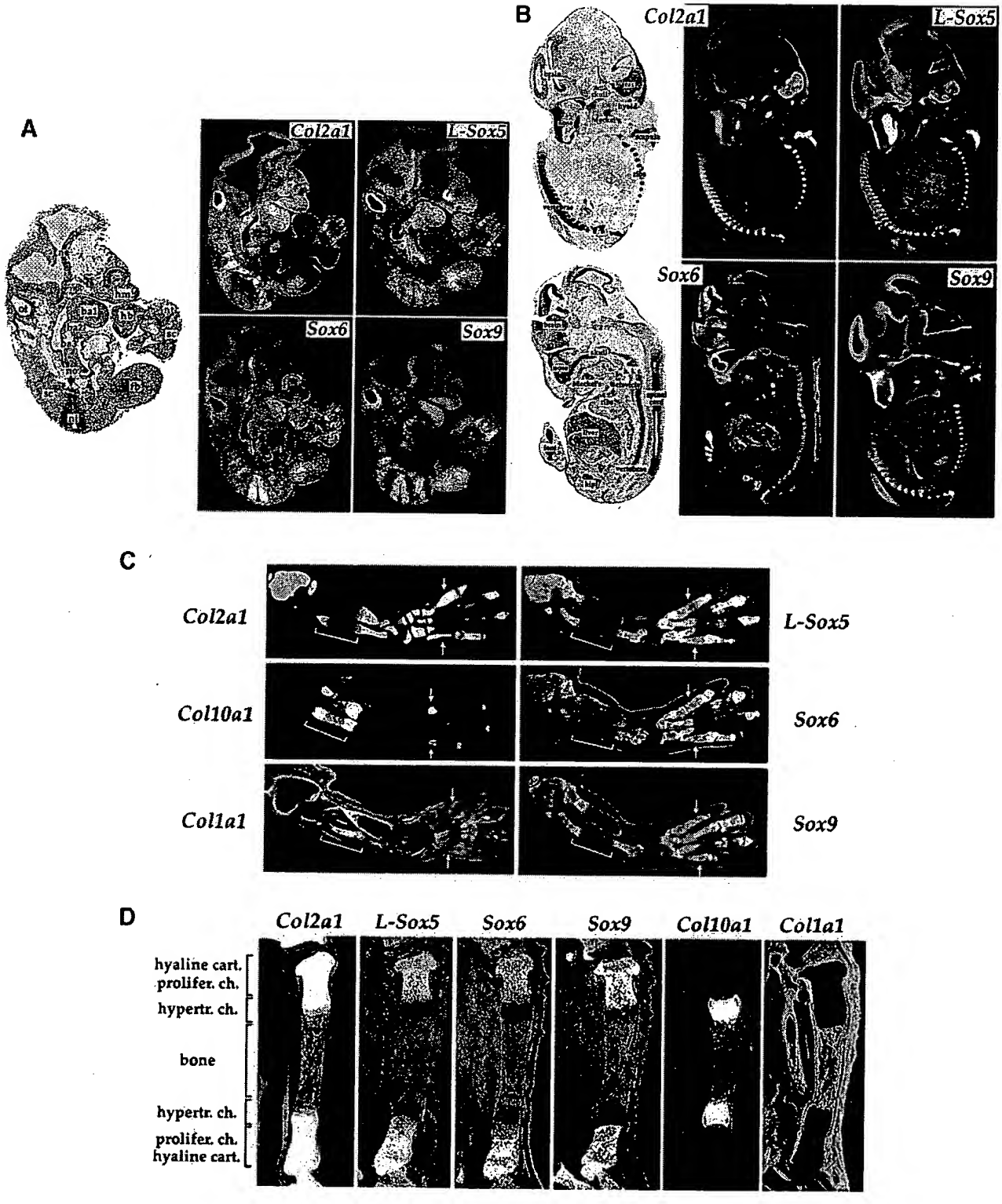
The long transcripts of Sox5 and Sox6 are coexpressed with Sox9 and Col2a1 during chondrogenesis in mouse embryos

Previous whole-mount *in situ* experiments showed that *Sox6* was expressed in the developing nervous system

Fig. 3. Transcripts for L-Sox5, Sox6 and Sox9 are coexpressed with *Col2a1* in chondrogenesis in mouse embryos. (**A**) *In situ* hybridization of sections through 10.5-day-old mouse embryos. A dark-field picture of a section hybridized with *Col2a1* probe was inverted to show the following: ba1-3, first to third branchial arches; fb, forelimb bud; hb, hindlimb bud; hm, head mesenchyme; ne, neural epithelium; no, notochord; nt, neural tube; ot, otic vesicle; and sc, sclerotome. All these sites expressed transcripts of type II collagen, L-Sox5, Sox6 and Sox9, as shown in dark-field pictures. Expression of *Col2a1* in hindlimb buds is not yet seen in 10.5-day-old embryos, in contrast to expression of the three *Sox* genes. (**B**) *In situ* hybridization of sagittal sections through 12.5-day-old mouse embryos. Dark-field pictures of sections hybridized with *Col2a1* and *Sox6* probes were inverted to show skeletal and non-skeletal structures; h.t.c., cartilage primordium of hyoid bone, thyroid and cricoid cartilages. Dark-field pictures show expression of transcripts for type II collagen, L-Sox5, Sox6 and Sox9 in these areas. (**C**) *In situ* hybridization of longitudinal sections through a forelimb of a 15.5-day-old mouse embryo. Adjacent sections were hybridized with collagen and Sox RNA probes. *Col10a1*, gene for α1(X) collagen; *Col1a1*, gene for pro-α1(I) collagen. Arrows point to the diaphysis of cartilages in the metacarpus, where chondrocytes become hypertrophic before giving way to osteoblasts. In these areas, chondrocytes are downregulating expression of the *Sox* genes and activating expression of *Col10a1*. A bracket underlines the diaphysis of the ulna, where ossification is more advanced than in the metacarpals: at each extremity, hypertrophic chondrocytes have turned off expression of the *Sox* genes and are switching from *Col2a1* to *Col10a1* expression; in the center, osteoblasts are expressing *Col1a1*. (**D**) *In situ* hybridization of longitudinal sections through a hindlimb of a 17.5-day-old mouse embryo. Adjacent sections were hybridized with collagen and Sox RNA probes. Brackets show different zones of cartilage and bone in the tibia. Chondrocytes in hyaline cartilage and proliferating chondrocytes in growth plates actively express *Col2a1* and transcripts for L-Sox5, Sox6 and Sox9; at a later stage of differentiation, hypertrophic chondrocytes no longer express the *Sox* genes, and *Col2a1* expression is being progressively replaced by *Col10a1* expression. Osteoblasts and cells in surrounding tissues are expressing *Col1a1*.

of mouse embryos; expression was high in early-stage embryos, but disappeared by embryonic day 12.5 (Connor *et al.*, 1995). To obtain information on *Sox6* and *Sox5* expression during chondrogenesis in mouse embryos, we performed a series of *in situ* experiments and compared the expression patterns of these two *Sox* genes with those of *Sox9* and *Col2a1*. For *Sox5*, we chose a probe that recognized the long transcript of *Sox5* but not the short transcript, since we were interested in sites of expression of the L-Sox5 protein only. For *Sox6*, two probes were tested that recognized either the long transcript only or both the long and short transcripts, since both transcripts appear to encode the same protein (Connor *et al.*, 1995; Takamatsu *et al.*, 1995); the two probes gave identical results (data not shown).

At about mid-stage embryogenesis (day 10.5 post-



coitum), mesenchymal cells form prechondrocytic condensations at different sites in the embryo. The sclerotomal components of somites contain precursor cells for the axial skeleton, the head mesenchyme and first and second branchial arches will generate part of the craniofacial skeleton, and the lateral plate mesoderm and limb buds will give rise to the appendicular skeleton. Transcripts for L-Sox5 and Sox6 were found in all these sites together with *Col2a1* and *Sox9* RNAs (Figure 3A; Cheah *et al.*, 1991; Wright *et al.*, 1995; Ng *et al.*, 1997; Zhao *et al.*, 1997). The four RNAs also co-localized in some non-chondrogenic sites such as brain, neural tube, otic vesicles and notochord.

At day 12.5 of embryonic development, cartilage is actively forming in all future cartilaginous and endochondral skeletal structures, including cartilages of the nose and ear, the thyroid, cricoid and hyoid cartilages, Meckel's cartilage, cartilages of the base of the skull, ribs, vertebrae, forelimbs and hindlimbs (Figure 3B). High levels of transcripts for L-Sox5 and Sox6 were found in all these structures, together with *Col2a1* and *Sox9* RNAs. Expression of the four genes was also seen in some areas of the brain and spinal cord. Transcripts for Sox6 and L-Sox5, but not Sox9, were also visible in liver and a few other non-cartilaginous areas.

In the limbs of 15.5- and 17.5-day-old embryos (Figure 3C and D), expression of *Col2a1* and of the three *Sox* genes was high in the cartilaginous templates of the radius and ulna, carpals, metacarpals and tibia, and in proliferating chondrocytes of growth plates. When chondrocytes became hypertrophic in growth plates, they activated expression of *Col10a1* whereas RNAs, for all three Sox proteins disappeared rapidly and simultaneously and RNAs for *Col2a1* disappeared more slowly. Only traces of *Col2a1* and *Sox* RNAs were found after cartilage was replaced by bone and expression of *Col1a1* was activated in osteoblasts.

In conclusion, RNAs for collagen II, L-Sox5, Sox6 and Sox9 were expressed simultaneously and at high levels from early stages of chondrogenesis in all cartilaginous sites in mouse embryos. Expression of the three *Sox* genes appeared to be inhibited just before *Col2a1* expression in hypertrophic chondrocytes. These results are consistent with a role for all three Sox proteins in the activation of *Col2a1* in chondrogenesis, and possibly also in the activation of other genes of the chondrogenic program.

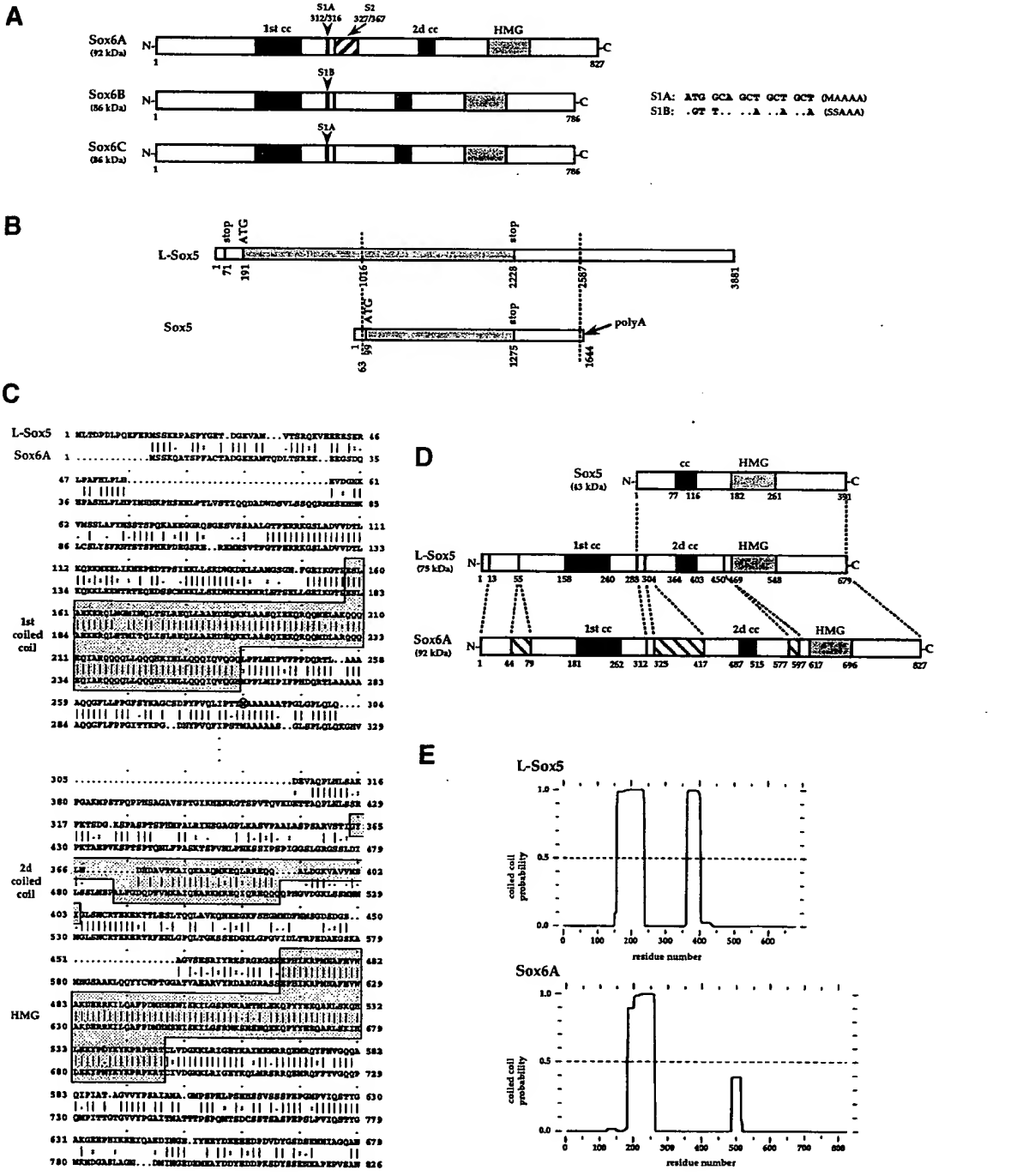
Comparison of Sox5, L-Sox5 and Sox6 cDNAs and polypeptides

Connor *et al.* (1995) and Takamatsu *et al.* (1995) previously isolated Sox6 cDNA species from adult mouse testis cDNA libraries. The cDNAs reported by the two groups encoded slightly different proteins, called Sox6 and Sox-LZ, respectively. For convenience, we have renamed them Sox6A and Sox6B. The proteins differ in two segments, most likely encoded by alternatively spliced exons (Figure 4A). Segment 1 encodes a MAAAA amino acid sequence (S1A) in Sox6A and a SSAAA sequence (S1B) in Sox6B, with different A codons being used in S1A and S1B. Segment 2 encodes a 41-amino-acid sequence and is present only in Sox6A. When total cDNA from primary chondrocytes was used in the polymerase chain reaction (PCR) with primers flanking the region of the S1 and S2 segments, the amplified products had the size expected for Sox6A (478 bp) and Sox6B (355 bp) products (data not shown). Sequencing of the PCR products indicated that the longer product contained S1A and S2, and was identical to Sox6A. The shorter product contained S1A and lacked S2. It therefore corresponded to a new form of Sox6, which we have named Sox6C (Figure 4A). In DNA-binding and transactivation assays, the three forms of Sox6 displayed identical activities (data not shown). These PCR data and additional sequencing of Sox6 cDNA clones from chondrocyte cDNA libraries suggested that the short and long transcripts of Sox6 both encoded protein isoforms with minor differences. Sox6 cDNA clones obtained from chondrocyte libraries contained an additional 3′ untranslated sequence at the 3′ end of the previously reported cDNAs from testis (data submitted to DDBJ/EMBL/GenBank). The longest sequence was 1.5 kb and accounts in part for the difference between the long and short transcripts of *Sox6*.

Fig. 4. Comparison of Sox5, L-Sox5 and Sox6 cDNAs and polypeptides. (**A**) Schematic comparison of Sox6A, Sox6B and Sox6C polypeptides. Three forms of Sox6 differed in segments S1 and S2. Sox6A and Sox6B are schematized according to the sequences reported by Connor *et al.* (1995) and Takamatsu *et al.* (1995), respectively. Sox6C, whose cDNA sequencing has not been completed, is schematized based on the assumption that it is identical to Sox6A and Sox6B outside of segments S1 and S2. Two coiled-coil domains (1st cc and 2d cc) and the HMG DNA-binding domain, are indicated. S1A and S1B segments are compared at the nucleotide level and, in parentheses, at the amino acid level. (**B**) Comparison of Sox5 and L-Sox5 cDNAs. Sequences available for Sox5 and L-Sox5 cDNAs are presented as blocks. Shaded areas indicate coding sequences, between the first in-frame ATG codon and the next stop codon. Sox5 and L-Sox5 cDNAs are identical in the segment delineated by the two dotted lines, but they differ totally on either sides of this segment. Numbers refer to nucleotide positions relative to the 5′ end. The Sox5 cDNA representation follows the sequence published by Denny *et al.* (1992). L-Sox5 cDNA is schematized according to a 3881 bp sequence obtained from overlapping clones. Both cDNAs must lack 5′ and/or 3′ untranslated sequences, as RNA transcript lengths were estimated to be ~2 kb for Sox5 and ~6.3 kb for L-Sox5. (**C**) Alignment of L-Sox5 and Sox6A amino acid sequences. The sequence of L-Sox5 (upper sequence), derived from its cDNA sequence, is compared with that of Sox6A (lower sequence) (Connor *et al.*, 1995). Numbers refer to amino acid positions in the proteins. Sequence alignment was generated using the GAP program from the Genetics Computer Group (GCG, Madison, WI). Dots were introduced within sequences to maximize alignment. Vertical lines denote amino acid identities. Double and single dots between sequences indicate amino acid changes with high and low degrees of conservation, respectively. The methionine translation initiation codon of the short form of Sox5 is circled in the L-Sox5 sequence (residue 288). Boxes outline two potential coiled-coil domains and the HMG DNA-binding domain. The sequence of Sox6A spanning residues 330–379 was omitted because it has no counterpart in L-Sox5. (**D**) Schematic comparison of Sox5, L-Sox5 and Sox6A polypeptides. Each protein is represented as a block between its N- and C-termini. HMG, HMG DNA-binding domain; cc, potential coiled-coil domain. Hatched boxes represent regions of >10 residues in Sox6A that do not exist in L-Sox5. Dotted lines link regions of similarity between the proteins. Numbers refer to the position of the residues marking domain limits. The predicted molecular weight of each protein is given in parentheses. (**E**) Delineation of potential coiled-coil domains in L-Sox5 and Sox6A. The amino acid sequences of L-Sox5 and Sox6A were analyzed with the Coilscan program from GCG using an unweighted matrix at a window size of 28 residues. The probability of each residue participating in coiled-coil formation is plotted against its position in the sequences. A score of 1.0 indicates a maximum probability. The first potential coiled coil involves residues 158–240 in L-Sox5 and residues 181–262 in Sox6A; the second potential coiled coil spans residues 364–403 in L-Sox5 and 488–515 in Sox6A.

The L-Sox5 cDNA that was reconstituted from overlapping clones contained a 2037 bp open reading frame that encodes a 679-amino-acid protein, with 287 amino acids N-terminal to, and in frame with, the 392-amino-acid Sox5 protein sequence (Denny *et al.*, 1992) (Figure 4B–D). It contained 189 bp of 5′ untranslated sequence, with an in-frame stop codon 120 bp upstream of the first ATG (data not shown). The first three in-frame methionine codons are surrounded by sequences homologous to the Kozak consensus and are therefore putative translation initiation codons (data not shown). Sox5 (Denny *et al.*, 1992) and L-Sox5 cDNAs share an uninterrupted 1571 bp sequence that includes the 1179 bp coding sequence of Sox5 (Figure 4B). Upstream and downstream of this region, the sequences available for the two cDNAs are totally different. Sox5 and L-Sox5 mature RNAs must therefore arise from differential splicing of primary transcripts in the 5′ and 3′ regions, and different promoter usage for the two RNAs cannot be excluded.

Previous comparison of the short Sox5 protein with Sox6A (Connor *et al.*, 1995) indicated an overall high degree of identity (61%), with a maximum of 93% in the HMG DNA-binding domain (Figure 4C and D). By comparison, the HMG domains of Sox5 and Sox6 are only 54 and 53% identical to that of Sry, respectively, and 50% and 49% identical to that of Sox9. The N-terminal



segment of L-Sox5 also has a high degree of sequence identity (72%) with the N-terminus of Sox6 (Figure 4C and D). Computer analysis of the protein sequences revealed two regions compatible with formation of coiled coils in L-Sox5 and Sox6 (Figure 4E) (Lupas, 1996). The more upstream coiled-coil domain is highly conserved between L-Sox5 and Sox6 (91% identity) and has a maximum probability score for coiled-coil formation of 1.0 in both proteins. It is particularly long, with 83 residues in L-Sox5 and 82 in Sox6, which implies that the coiled coil may complete almost 24 helical turns. It includes in its N-terminal part the leucine zipper previously identified in Sox6 (Connor *et al.*, 1995; Takamatsu *et al.*, 1995) and a glutamine-rich domain in its C-terminal part, referred to as a Q box (Kido *et al.*, 1998) (Figure 4C). The more downstream coiled-coil domain is located in the sequence of L-Sox5 that is shared with Sox5. This domain is shorter, with 40 residues in Sox5 and 29 in Sox6. It has a maximum probability score of 1.0 in Sox5 but a low-probability score of 0.39 in Sox6. It is only moderately conserved between the two proteins (59% identity). No region of homology with Sox9 was found outside the HMG domain.

In conclusion, the striking identity of L-Sox5 and Sox6 in the first coiled-coil domain and in the HMG domain strongly suggests that each of these domains must serve one or several important functions. The presence of these domains in both proteins and the otherwise overall high degree of identity between the two proteins (67%) suggest that L-Sox5 and Sox6 may play similar roles *in vivo*.

Dimerization of L-Sox5 and Sox6 highly stabilizes binding to adjacent HMG sites on DNA

L-Sox5 and Sox6 bound to the 2HMG probe much more efficiently than to the 1HMG probe (Figure 5A). In contrast, SOX9 bound with similar efficiency to both probes. This observation, the presence of potential coiled coils in L-Sox5/Sox6 and the ability of Sox6 to homodimerize (Takamatsu *et al.*, 1995) led to the hypothesis that dimerization of the proteins through their coiled coils might stabilize their binding to adjacent DNA-binding sites.

To test this hypothesis, various deletion forms of L-Sox5 were synthesized *in vitro* and treated with glutaraldehyde (Figure 5B), which has the ability to cross-link interacting polypeptides (Wong, 1991). After separation by SDS–PAGE (Figure 5C), polypeptides with the expected M_r were seen for each full-length and deleted protein species in samples treated with no glutaraldehyde. Upon treatment with glutaraldehyde, monomeric forms of Sox6 and L-Sox5 were less abundant, and polypeptide species appeared that had the apparent M_r of homodimers. Similarly, the 151/679 and 32/437 deletions formed cross-linked species with an M_r consistent with dimerization. In these deletions, the two coiled-coil domains were intact. Deletion 213/679, short Sox5 and deletion 32/221–304/437, all of which had the first coiled-coil domain partially or totally deleted, did not form specific cross-linked species, nor did SOX9. These results indicated that the first coiled-coil domain of L-Sox5, like that of Sox6 (Takamatsu *et al.*, 1995), was involved in specific protein–protein interactions, most likely protein homodimerization. The second coiled-coil domain of L-Sox5, which is present in 213/679, short Sox5 and deletion 32/221–304/437, appeared to be unable to mediate protein dimerization by itself. It might, however, have a role in stabilizing protein dimerization through the first coiled-coil domain.

In EMSA (Figure 5D), deletion 151/679 bound DNA as efficiently as L-Sox5 (lanes 2 and 3), but deletion 213/679 did not (lane 4), nor did Sox5 (lane 5). The first coiled-coil domain of L-Sox5 (residues 158–240) was still intact in 151/679 but largely deleted in 213/679. It appeared therefore to be involved in the high affinity of L-Sox5 for the 2HMG probe. A protein truncated in the C-terminus (32/437) did not bind DNA (lane 6), an expected result given that the HMG domain was deleted.

When L-Sox5 was mixed with deletion 151/679, the respective complexes of the two proteins formed, but a third abundant complex also formed whose migration level indicated that it was likely to involve one molecule of each of the two proteins (lane 9). A similar intermediate complex also formed with Sox6 and 151/679 (lane 15). This result strongly suggested that L-Sox5 binds the 2HMG probe as a homodimer or heterodimer with Sox6. On the basis of its DNA-binding properties, a similar conclusion can be made for Sox6.

When L-Sox5 was mixed with deletion 213/679 (lane 10), only the complex of L-Sox5 homodimer with DNA formed, indicating that the coiled-coil domains of two molecules of L-Sox5 were needed for efficient binding to DNA. Also, when L-Sox5 was mixed with deletion 32/437 (lane 12), no heterodimers bound to the 2HMG probe, indicating that the two C-termini of L-Sox5 molecules (which included the HMG domain) were required to bind DNA.

Together, these results suggest a model (Figure 5E) in which binding of two molecules of L-Sox5 and Sox6 to adjacent HMG sites on DNA is highly stabilized by protein dimerization through the coiled-coil domains.

Cooperative activation of chondrocyte-specific Col2a1 constructs by L-Sox5, Sox6 and SOX9

The ability of L-Sox5, Sox6 and SOX9 to activate *Col2a1* reporter constructs in non-chondrogenic cells was tested using constructs in which *Col2a1* enhancer segments were placed in an intron downstream of the promoter, as is the case in the endogenous *Col2a1* gene.

A construct with a 309 bp *Col2a1* promoter (p309; Figure 6A) was previously shown to be inactive in chondrocytes of transgenic mice (Zhou *et al.*, 1995). In transient transfection of 10T1/2 cells, this construct was slightly but reproducibly activated by L-Sox5/Sox6 but not by SOX9, and no cooperation occurred among the three Sox proteins (Figure 6B). A similar construct that included four tandem copies of the 48 bp enhancer [p309–(4x48); Figure 6A] was specifically expressed in chondrocytes both in transgenic mice (Lefebvre *et al.*, 1996) and in transient transfection (data not shown). In non-chondrogenic cells, this construct was not activated by L-Sox5, Sox6 or a combination of L-Sox5 and Sox6 at a higher level than the p309 construct, and it was moderately activated by SOX9 (Figure 6C). Interestingly, when SOX9 was co-expressed with L-Sox5, Sox6, or both L-Sox5 and Sox6, a higher activation occurred than with any Sox protein alone, demonstrating cooperativity between SOX9 and L-Sox5/Sox6.

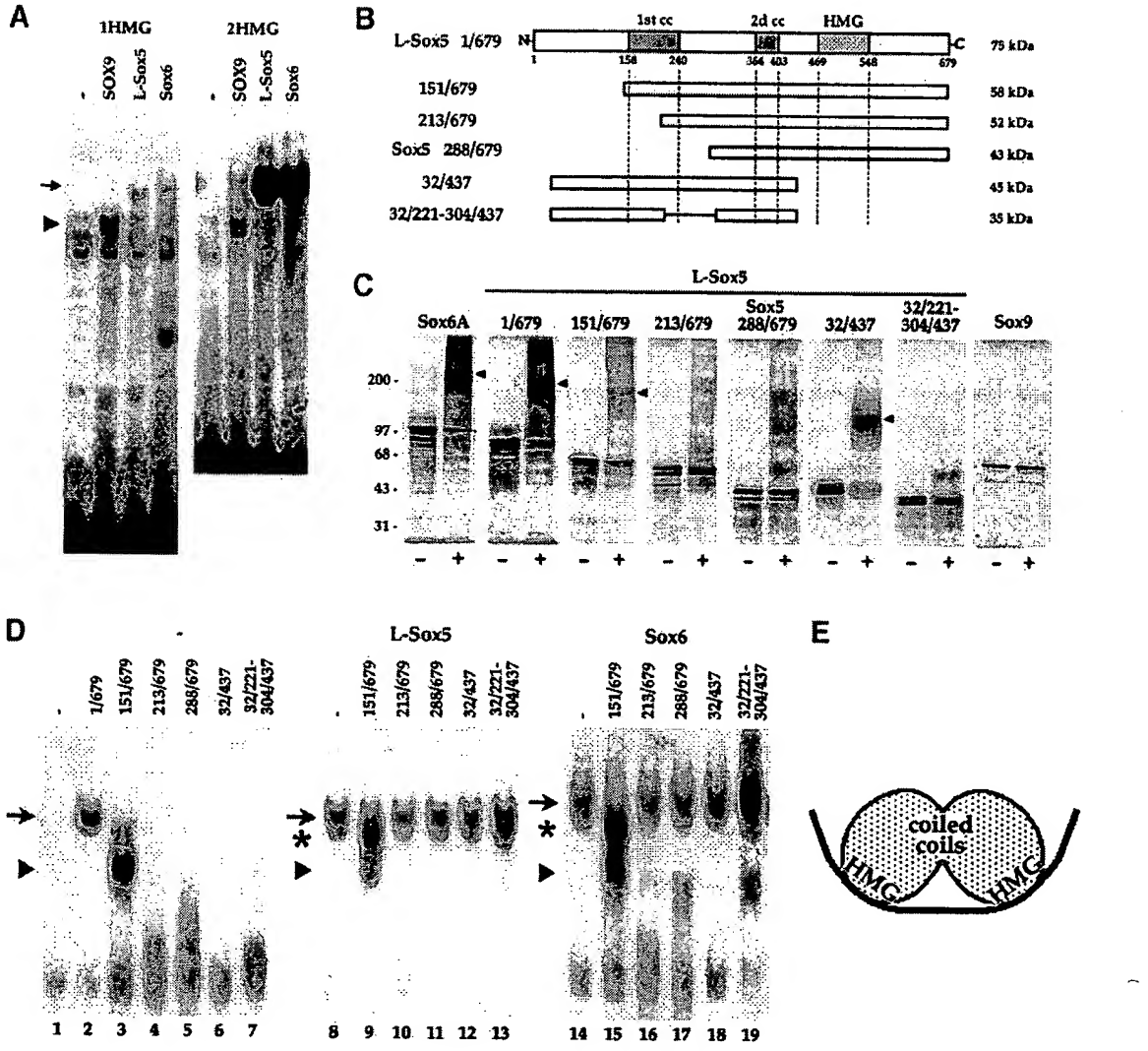


Fig. 5. Dimerization of L-Sox5 and Sox6 stabilizes binding to adjacent HMG sites. **(A)** L-Sox5 and Sox6 bind the 2HMG probe more efficiently than the 1HMG probe. Extracts of 10T1/2 fibroblasts transfected with empty (–), SOX9, L-Sox5 or Sox6 expression plasmids were incubated with the 1HMG probe or the 2HMG probe. The two probes had the same radioactivity. Arrow, L-Sox5–Sox6 complexes with DNA and slow-migrating SOX9–DNA complex. Arrowhead, fast-migrating SOX9–DNA complex; this complex was often seen as a doublet. **(B)** Deletions in L-Sox5. L-Sox5 full-length protein is schematized as described in Figure 4D. Truncated proteins were named by the first and last residues of L-Sox5 that they contain. In 32/221–304/437, the sequence 222/303 in 32/437 was deleted. **(C)** The first coiled-coil domain of L-Sox5 and Sox6 is involved in protein–protein interactions. Sox6, L-Sox5, truncated L-Sox5 polypeptides and SOX9 were synthesized *in vitro* with [^{35}S]methionine and then incubated with (+) or without (–) glutaraldehyde. Autoradiographs are shown after polypeptide separation by SDS–PAGE. Arrowheads, glutaraldehyde-induced cross-linked polypeptides. The M_r of protein standards is indicated ($\times 10^3$). **(D)** L-Sox5–Sox6 bound the 2HMG probe as dimers. Extracts of 10T1/2 fibroblasts transfected with empty (–), L-Sox5 or Sox6 expression plasmids were preincubated with products of *in vitro* transcription/translation obtained with plasmids encoding no protein (–) or one of the truncated L-Sox5 proteins (indicated on top of the lanes). EMSA was performed with the 2HMG probe. Arrows, complexes of L-Sox5 and Sox6 with DNA. Arrowhead, complex of 151/679 with DNA. Star, heterocomplexes of 151/679, and L-Sox5 or Sox6 with DNA. **(E)** Model. Two molecules of L-Sox5/Sox6 (ovals) form a highly stable complex with DNA upon binding to two adjacent recognition sites on DNA (thick curved line). The two protein molecules dimerize through their coiled-coil domains and may induce a strong bend of DNA at the sites of binding.

Similarly, a p309–(2x231) construct, which harbored two copies of a 231 bp *Col2a1* chondrocyte-specific enhancer (Lefebvre *et al.*, 1996), including the 48 bp element, was activated at a higher level by the three Sox factors together than by each Sox protein individually (Figure 6D). Deletion of a 10 bp sequence in the 231 bp enhancer, which corresponded to the 3′ end of the 48 bp element, abolished enhancer activity in chondrocytes (Lefebvre *et al.*, 1997) and also transactivation by the three Sox factors (Figure 6D). In contrast to L-Sox5/Sox6, Sox4 and the short form of Sox5 did not activate the p309-(2×231) construct, nor did they cooperate with SOX9 to generate a high-level transactivation of the construct in fibroblasts (Figure 6E).

Together these results indicated that L-Sox5 and Sox6 significantly enhanced transactivation of *Col2a1* constructs by SOX9. L-Sox5 and Sox6 appeared to function similarly, whereas the short form of Sox5 was inactive.

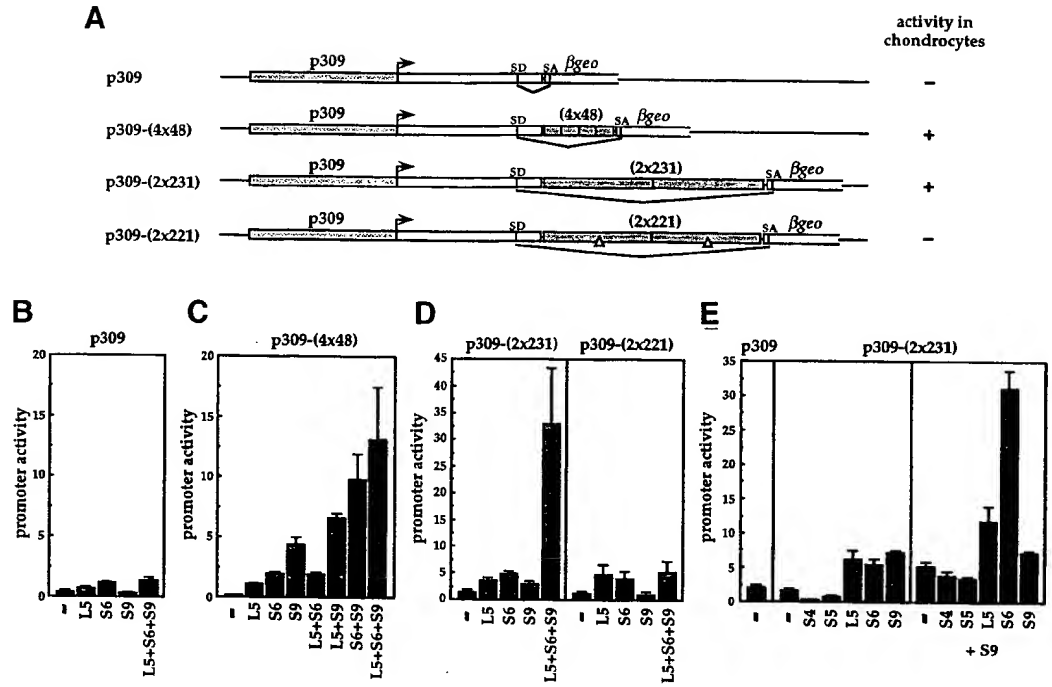


Fig. 6. Cooperative activation of chondrocyte-specific *Col2a1* promoter–enhancer constructs by L-Sox5, Sox6 and SOX9. (**A**) Schematic of *Col2a1* constructs. The βgeo reporter gene was driven by a 309 bp (p309; –309/+308) *Col2a1* promoter. *Col2a1* enhancer segments were four tandem copies of the 48 bp intron-1 element (4x48), two tandem copies of a 231 bp element (2x231) or two tandem copies of a 231 bp element in which 10 bp (triangles) were deleted (2x221). The deletion corresponded to the 3′ end of the 48 bp element. Enhancers were cloned downstream of the *Col2a1* exon 1 in an intron delimited by a splice donor (SD), the proximal 70 bp of the *Col2a1* intron 1 and a splice acceptor (SA). The '–' and '+' symbols indicate whether the constructs were inactive or active, respectively, in chondrocytes. (**B**) Activation of p309. 10T1/2 fibroblasts were transfected with p309 and 900 ng of expression plasmids. These included empty vector (–) and, as indicated, 300 ng of L-Sox5 (L5), Sox6 (S6) and SOX9 (S9) plasmids. (**C**) Activation of p309–(4x48). Similar experiment as in (B) but with p309–(4x48) instead of p309. (**D**) Activation of p309–(2x231) and p309–(2x221). 10T1/2 fibroblasts were transfected with p309–(2x231) or p309–(2x221), and either 600 ng of empty (–) L-Sox5, Sox6 or SOX9 plasmid, or 200 ng of each of the L-Sox5, Sox6 and SOX9 plasmids. (**E**) No activation of p309–(2x231) by Sox4 and Sox5. 10T1/2 fibroblasts were transfected with p309 or p309–(2x231), and either 600 ng of empty (–), Sox4 (S4), Sox5 (S5), L-Sox5, Sox6 or SOX9 plasmid, or 300 ng of SOX9 plasmid and 300 ng of empty or SOX plasmid.

Cooperative activation of chondrocyte-specific genes by L-Sox5, Sox6 and SOX9

The ability of L-Sox5/Sox6 and SOX9 to activate the endogenous *Col2a1* gene was tested in several cell types by Northern blot analysis after transient transfection with Sox expression plasmids. As described previously (Lefebvre *et al.*, 1997), 10T1/2 cells spontaneously expressed *Col2a1*, but at a much lower level than did differentiated chondrocytes (Figure 7A). Following transfection of Sox plasmids, Sox RNAs and proteins accumulated in large amounts in the cells within 24 h, but rapidly disappeared during the next 48 h (Figure 7A). A significant increase in the *Col2a1* mRNA level was observed 24 h after transfection of either SOX9 or L-Sox5/Sox6 plasmids (Figure 7A and B). Depending on the experiments, SOX9 was either as potent as, or more potent than, L-Sox5/Sox6. The increase in *Col2a1* RNA was transient and no longer detectable after 48 h (Figure 7A), and was therefore concomitant with high levels of Sox proteins. The same results were observed whether L-Sox5 and Sox6 were tested alone or together, indicating that they had redundant activities (data not shown). No significant increase in *Col2a1* expression was observed when the cells were transfected with an expression plasmid for Oct-1, a POU-domain transcription factor capable of binding to the 48 bp *Col2a1* enhancer (data not shown). This result demonstrated the specificity of Sox protein activity. Interestingly, coexpression of SOX9 and L-Sox5/Sox6 resulted in a much higher activation of *Col2a1* expression than when each Sox protein was expressed individually. Similar results were obtained with MC615 cells (Figure 7B), which were tested after repeated passages in culture when expression of chondrocyte markers was severely reduced or lost. Their low level of *Col2a1* expression was slightly stimulated upon expression of SOX9 alone or L-Sox5/Sox6, but it was strongly stimulated by coexpression of the three Sox proteins. When the cell culture medium was supplemented with bone morphogenetic protein 2 (BMP-2), a cytokine known to promote chondrogenesis (Reddi, 1998), *Col2a1* RNA level also increased. In this condition also, overexpression of the three Sox factors highly stimulated *Col2a1* expression. When cells that did not spontaneously express *Col2a1* were tested, such as skin primary fibroblasts from newborn mice, BALB/3T3 fibroblasts and COS cells, no induction of *Col2a1* expression was detected following transfection of Sox protein expression plasmids (data not shown).

Activation by L-Sox5/Sox6 and SOX9 of other genes expressed in chondrocytes along with *Col2a1* was also examined (Figure 7B). The *aggrecan* gene, which encodes the protein core of a large aggregating proteoglycan found in abundance in cartilage, was weakly expressed by dedifferentiated MC615 cells, but overexpression of the three Sox proteins led to a clear increase in its RNA level.

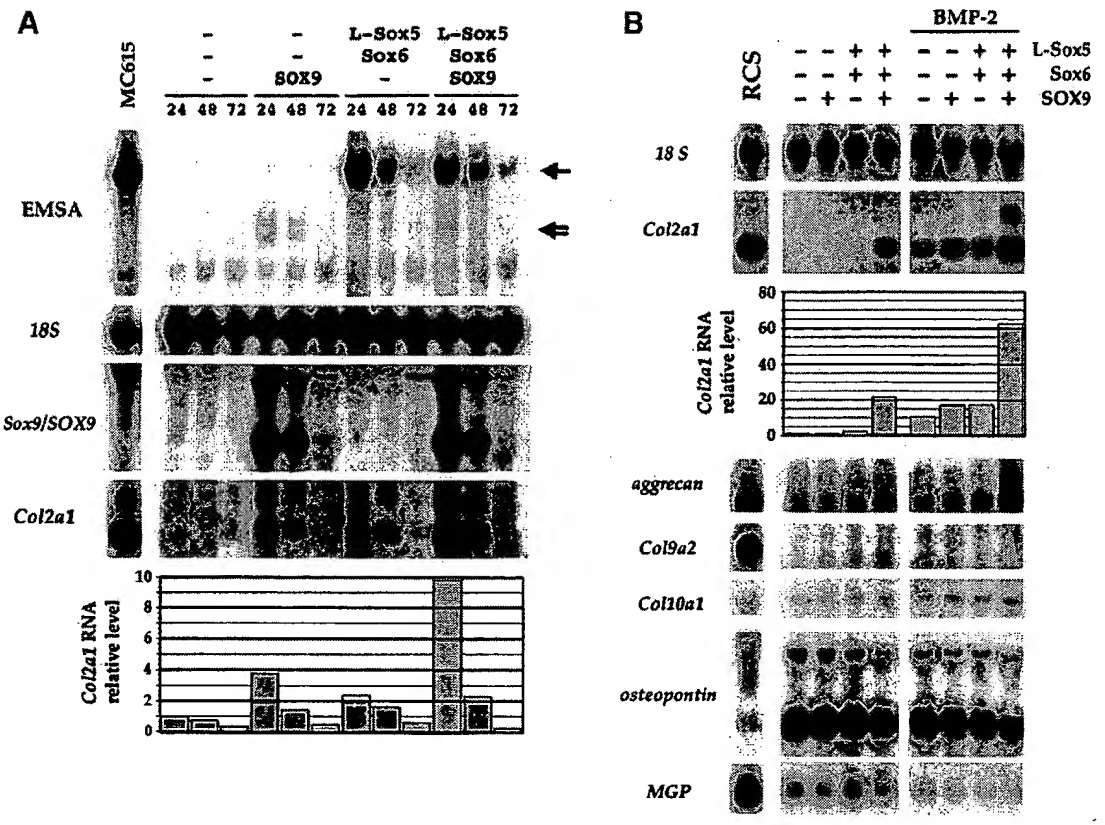


Fig. 7. Cooperative activation of chondrocyte-specific genes by L-Sox5, Sox6 and SOX9. (A) Transient transfection of 10T1/2 cells. L-Sox5, Sox6 and/or SOX9 expression plasmids were transfected as indicated. Cells were harvested 24, 48 or 72 h after the start of transfection. Total cell extracts were used in EMSA with the 2HMG probe. Single-tailed arrow, L-Sox5–Sox6–DNA complexes; double-tailed arrow, fast-migrating SOX9–DNA complexes. The weak intensity of SOX9–DNA complexes compared with L-Sox5–Sox6–DNA complexes reflects a less-efficient binding of SOX9 to the 2HMG probe. Total RNA was hybridized in Northern blots with probes for 18S rRNA (reference for RNA loading), *Sox9* and *Col2a1*. Differentiated MC615 cells at an early passage are shown in the left lane as a reference for chondrocytic cells. Endogenous *Sox9* RNA forms a band at the top of the panel; *SOX9* RNAs expressed from the transfected plasmid run as an intense band migrating faster. The more intense signal seen at the level of endogenous *Sox9* RNA in cells transfected with *SOX9* may be due either to increased expression of *Sox9* or to larger transcripts from *SOX9* plasmid. The exposure of the autoradiograph of the *Col2a1* blot was eight times as short (3 h) for MC615 cells as for 10T1/2 cells. The intensity of hybridization signal for *Col2a1* RNA in the different culture conditions is plotted as fold increase over the signal given by the control culture 24 h after transfection. (B) Transfection of MC615 cells. Cells that had essentially lost their chondrocytic phenotype by repeated passaging were transfected with Sox expression plasmids, as indicated. BMP-2 was added at the start of transfection. Cells were harvested 24 h later and RNA analyzed in Northern blot with various probes, as indicated. RNA from RCS cells was used as a reference for differentiated chondrocytes. The intensity of the *Col2a1* hybridization signal in the different conditions is plotted as fold increase over the signal given by the control culture in the absence of BMP-2. Hybridization signals obtained with an 18S rRNA probe are shown as a reference for RNA loading. The exposure of the autoradiograph of the *aggrecan* blot was five times as short for RCS cells (3 h) as for 10T1/2 cells.

RNA for *Col9a2*, which codes for the α2 chain of type IX collagen, was undetectable in MC615 cells and not induced by any of the three Sox protein expression plasmids. RNAs for *aggrecan* and *Col9a2* were not detectable in 10T1/2 cells, even after transfection of the three Sox protein expression plasmids (data not shown).

RNA levels of genes that are expressed in chondrocytes but not in parallel with *Col2a1* were examined in order to test the specificity of gene transactivation by the three Sox proteins. *Col10a1*, a characteristic marker of hypertrophic chondrocytes, was expressed at low levels in MC615-dedifferentiated cells, but its expression was not affected by transfection of Sox protein expression plasmids. The genes for matrix Gla protein (MGP) and osteopontin, two extracellular matrix proteins produced by chondrocytes and also some other cell types, were expressed in MC615 cells, but transfection of the three Sox factors did not significantly affect their RNA levels (Figure 7B).

In conclusion, L-Sox5/Sox6 and SOX9 were found to stimulate cooperatively expression of *Col2a1* and also *aggrecan*, two major markers of chondrocytes. These data strongly suggest that the three sox proteins together control important aspects of the chondrocyte phenotype.

Discussion

We have shown here that a new form of Sox5 (L-Sox5) and Sox6, which both are dimeric Sox proteins that preferentially bind to adjacent HMG sites rather than to isolated sites, are coexpressed with Sox9 during chondrogenesis, efficiently bind to several HMG-like sites in the 48 bp chondrocyte-specific *Col2a1* enhancer, and cooperate with SOX9 in activating the chondrocyte marker gene *Col2a1*.

L-Sox5 differs from Sox5 by an additional 287 amino-acid sequence at the N-terminus. L-Sox5 and Sox6 show a striking degree of identity in the HMG DNA-binding

domain located in the C-terminal part and in a coiled-coil domain located in the N-terminal part. This coiled-coil domain has been shown previously to mediate homodimerization of Sox6 (Takamatsu *et al.*, 1995), and we have shown that L-Sox5 also dimerizes through this domain, either with itself or with Sox6. Sox12, Sox13 and Sox23 (Komatsu *et al.*, 1996; Kido *et al.*, 1998; Yamashita *et al.*, 1998) also feature a similar coiled-coil domain and their HMG domain is much more similar to those of L-Sox5 and Sox6 than to those of Sry, Sox9 and other Sox family members. Based on these similarities, the five Sox proteins are classified as Sox subgroup D (Wright *et al.*, 1993; Yamashita *et al.*, 1998). We have presented strong evidence that dimerization of L-Sox5/Sox6 highly stabilizes binding to DNA at adjacent recognition sites. Indeed, L-Sox5 and Sox6 bound to an element harboring two HMG sites much more efficiently than to an element harboring a single site. Two molecules of Sox polypeptides were binding to one molecule of 2HMG probe, and both the HMG and coiled-coil domains of the two polypeptide molecules were required for efficient DNA binding. SOX9, which does not homodimerize, did not bind to the 2HMG probe more efficiently than to the 1HMG probe. The complementary roles of the coiled-coil and HMG domains of L-Sox5/Sox6 in DNA binding probably account for the high degree of conservation of both domains in D-Sox family members. It also implies that target genes for these Sox proteins must harbor pairs of HMG binding sites with a configuration compatible with binding of D-Sox protein dimers.

Transcripts for L-Sox5 and Sox6 were expressed along with *Sox9* in all prechondrogenic areas and cartilages during mouse embryonic development. Expression of all three *Sox* genes was inhibited when chondrocytes became hypertrophic in growth-plate cartilages; in these cells expression of the chondrocyte marker gene *Col2a1* is also downregulated. *Sox9*, *Sox5* (transcript for L-Sox5) and *Sox6* were also expressed in some non-cartilaginous sites, such as notochord, otic vesicles and some areas of the brain, but they were not coexpressed where *Col2a1* was not expressed. In cell cultures, the three *Sox* genes were coexpressed only in primary chondrocytes and chondrocytic cell lines, both of which highly expressed *Col2a1*, and a sharp decrease in the three *Sox* RNA levels accompanied loss of *Col2a1* expression during chondrocyte dedifferentiation. We have also shown that the three Sox proteins were present in chondrocytes.

Our data indicate that when SOX9 and L-Sox5/Sox6 were transfected individually in 10T1/2 and MC615 cells, they only produced a modest increase in expression of the endogenous *Col2a1* gene. However, upon cotransfection, cooperativity occurred among the three Sox proteins, leading to expression levels of *Col2a1* that were an order of magnitude or more higher than in control cells. Expression of *aggrecan* was also significantly increased in MC615 cells transfected with the three Sox protein expression plasmids. The two classes of Sox proteins, L-Sox5/Sox6 and Sox9, are therefore able to cooperate functionally with each other in the activation of both *Col2a1* and *aggrecan*. These results, together with the strong correlation that exists between expression of *Col2a1*, *aggrecan* (Glumoff *et al.*, 1994) and the three Sox genes during chondrogenesis in mouse embryos, support the notion that the three Sox proteins may also play a role in the activation of *Col2a1*, *aggrecan* and possibly other chondrocyte marker genes *in vivo*.

A 48 bp element in the first intron of *Col2a1* was previously shown to be sufficient to direct expression of a reporter gene in cartilage of transgenic mice and to contain sites essential for the activity of longer *Col2a1* enhancer segments in chondrocytes (Lefebvre *et al.*, 1996; Bell *et al.*, 1997; Zhou *et al.*, 1998). This element is therefore likely to be involved in *Col2a1* expression in chondrocytes. Sox9, L-Sox5 and Sox6 bound to the 48 bp enhancer at four HMG-like sites, three of which were demonstrated to be necessary for enhancer activity in chondrocytes (Lefebvre *et al.*, 1996; Bell *et al.*, 1997; Zhou *et al.*, 1998), whereas the role of the fourth has not been tested. On their own, L-Sox5/Sox6 were weak activators of *Col2a1* enhancer constructs. However, they efficiently cooperated with SOX9 in the activation of the enhancer, leading to a level of activation that was several-fold higher than when each Sox protein was transfected individually. The promoter–enhancer configuration in these constructs was similar to that of the endogenous *Col2a1* gene, in which the chondrocyte-specific enhancer is located in the first intron downstream of the transcription start site. L-Sox5/Sox6 were able to contact all four HMG-like recognition sites of the 48 bp *Col2a1* enhancer. They probably bound as dimers, as their complexes with the enhancer and with the 2HMG probe migrated at the same level. Even though the binding sites for L-Sox5/Sox6 in the 48 bp enhancer are different from the preferred binding sites of the short Sox5 (AACAAT and AACAAAG; Denny *et al.*, 1992), our experiments indicate that L-Sox5/Sox6, as well as SOX9, efficiently bound to the enhancer. It is possible that the proximity of several HMG-like sites in the enhancer was favorable to cooperativity between the two types of Sox proteins in achieving transcriptional activation.

Together the three Sox factors enhanced *Col2a1* and *aggrecan* expression in cells that expressed low levels of these genes, but they did not induce *Col2a1* expression in cells in which the gene was silent, even though they activated *Col2a1* constructs at high levels in transient transfection of these same cells. It is possible that additional factors or coactivators may be needed to open the chromatin of chondrocyte-specific genes that were silent in the cells that were tested or that these genes were inactivated by other epigenetic mechanisms. Bell *et al.* (1997) reported that Sox9 was capable of activating *Col2a1* in some ectopic sites of transgenic mouse embryos. It is possible that the *Col2a1* gene is not repressed in mouse embryos as it might be in tissue culture cells or that the ectopic sites in which Sox9 was able to activate *Col2a1* contained factors that allowed Sox9 to activate *Col2a1*.

Denny *et al.* (1992) reported that Sox5 was unable to activate transcription from a minimal promoter linked to multimerized Sox5 binding sites. In our experiments, L-Sox5/Sox6 were able to activate *Col2a1* constructs whereas Sox5 was not. The more efficient binding of L-Sox5/Sox6 to DNA, compared with that of Sox5, is sufficient to explain why L-Sox5/Sox6 transactivated whereas Sox5 did not. But it is also possible that the N-terminus of L-Sox5/Sox6 harbors one or more domains

involved in transactivation. Takamatsu *et al.* (1995) reported that full-length Sox6 was unable to transactivate a reporter construct containing four copies of an HMG-binding site. Transactivation and DNA binding occurred upon deletion of the leucine zipper of the protein. In our experiments, L-Sox5/Sox6 bound to the *Col2a1* enhancer and transactivated *Col2a1* constructs as full-length proteins. Differences between the two studies may be due to differences in DNA targets. It may be that the sequence of the *Col2a1* enhancer, which is a potential target of L-Sox5/Sox6, the distance between HMG-like sites and eventually the presence of binding sites for additional activators are essential for L-Sox5/Sox6 binding to DNA and transactivation function.

HMG-domain proteins have been shown to participate as architectural factors in transcriptional activation of several genes (Grosschedl *et al.*, 1994; Werner and Burley, 1997). By their ability to bend DNA, a property also demonstrated for Sox5 and SOX9 (Connor *et al.*, 1994; Lefebvre *et al.*, 1997), they facilitate interactions between proteins bound at non-adjacent DNA sites and thereby promote the assembly of multiprotein–enhancer complexes (Giese *et al.*, 1995; Pevny and Lovell-Badge, 1997). It is possible that L-Sox5/Sox6 have such a role, eventually with Sox9, in organizing an enhancer–protein complex and also in bringing this complex close to the basal transcriptional machinery, which in *Col2a1* is 2.2 kb upstream of the enhancer. But the function of Sox9 is probably not limited to an architectural role, as it was shown to have a potent transactivation domain (Südbeck *et al.*, 1996; Lefebvre *et al.*, 1997; Ng *et al.*, 1997).

The function of L-Sox5/Sox6 *in vivo* is not known. The ability of L-Sox5/Sox6 to cooperate with SOX9 in *Col2a1* activation and the strong correlation between expression of L-Sox5/Sox6 and chondrogenesis suggest that mutations in the *SOX5* and *SOX6* genes might result in cartilage malformation diseases of still unknown causes. However, because of a possible redundancy of these two highly similar factors, a mutation in only one of their genes might cause only mild or no skeletal abnormalities. Although L-Sox5 and Sox6 belong to the same family of DNA-binding proteins as Sox9, and present the same expression pattern as Sox9 in chondrogenesis, it is unlikely that they play the same role as Sox9 because they differ substantially from Sox9 in DNA-binding and transactivation properties. The severe phenotype of patients with campomelic dysplasia, in which mutations in SOX9 are heterozygous, also strongly suggests that no other protein with a function that overlaps that of SOX9 exists in chondrocytes. Our data strongly suggest that Sox9 and L-Sox5/Sox6 represent two different subclasses of Sox proteins that are coexpressed during chondrogenesis, where they have distinct, complementary roles in the activation of important chondrocyte phenotype markers such as *Col2a1*.

Materials and methods

cDNA cloning

cDNA libraries were made from primary chondrocytes of ribs of newborn mice. Total RNA was isolated from cells cultured for 2–3 days (Lefebvre *et al.*, 1994). Poly(A)$^+$ RNA was purified using the Poly(A) Quick mRNA isolation kit from Stratagene (La Jolla, CA). Synthesis of double-stranded cDNA and ligation of adaptors were performed using the Superscript Choice system from Gibco-BRL (Gaithersburg, MD). Priming was performed with a mixture of oligo-dT and random hexamers. One expression library was made in the λgt11 phage vector and another in the λTriplEx phagemid vector from Clontech (Paolo Alto, CA). λ DNA was packaged with Gigapack III Gold Packaging extract (Stratagene). Southwestern screening was performed according to the method of Vinson *et al.* (1988) and Singh *et al.* (1989), and Clontech's instructions, after denaturation of filters with guanidine hydrochloride. Filters were preincubated for 30 min in incubation buffer (20 mM HEPES pH 7.9, 10% glycerol, 0.1% Nonidet P-40, 0.5 mM EDTA, 1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin and 0.5 mM DTT) and further incubated for 2 h in new buffer, supplemented with 50 fmol/ml of ^{32}P-labeled 2HMG probe and 1 μg/ml poly(dG–dC) or poly(dI–dC). Filters were washed four times for 1 min in incubation buffer and autoradiographed. New segments of L-Sox5 and Sox6 cDNAs were sequenced on both strands. A fragment of *Sox6* cDNA was amplified by PCR using primers encompassing two *Hin*cII restriction sites in the coding sequence and first-strand cDNA from mouse chondrocytes or adult testis. PCR products were electrophoresed in agarose gel, eluted and sequenced.

Electrophoretic mobility shift assay (EMSA)

EMSAs were performed as described previously (Lefebvre *et al.*, 1997) using a 1HMG or *Col2a1* 48 bp probe (Lefebvre *et al.*, 1997), or a 2HMG probe. The latter probe consisted of two tandem repeats of the 1HMG probe with *Bam*HI and *Bgl*II-cleaved sites at the 5′ and 3′ ends, respectively, and with the two repeats linked by the sequence GGATCT. Extracts from transfected fibroblasts were made in 100 mM potassium phosphate buffer, pH 7.2, containing 0.2% Triton X-100. Proteins were synthesized *in vitro* using the TNT T7 Quick Coupled Transcription/translation system (Promega, Madison, WI) and the expression plasmids described below.

Antibody preparation and Western blotting

Antisera were raised in rabbits (Genosys Biotechnologies, The Woodlands, TX) using keyhole limpet hemocyanin conjugated to a peptide homologous to the C-terminus of Sox9 (Bridgewater *et al.*, 1998) or corresponding to a sequence in the C-terminus of either Sox5 (PDVDYGSDSENHIAG) or Sox6 (PKSDYSSENEAPEPV). Specific antibodies were purified as described previously (Bridgewater *et al.*, 1998). Purified antibodies (~0.1 mg/ml) were dialyzed against phosphate-buffered saline. Western blots were prepared as described previously (Lefebvre *et al.*, 1997) with Sox protein antibodies diluted 1:1000.

Probes for Northern blotting and in situ hybridization

A *Nco*I–*Nru*I 552 bp fragment of mouse *L-Sox5* cDNA, located in the 5′ end of the coding sequence, specifically hybridized with the long transcript of *Sox5*. A *Nco*I 448 bp fragment of *Sox5* cDNA, which included the HMG box, specifically hybridized with the short and long transcripts of *Sox5*. A *Bgl*II–*Hin*dIII 454 bp fragment of *Sox6* cDNA, located 1.3 kb downstream of the stop codon, specifically hybridized with the long transcript of *Sox6*. An *Acc*I–*Pst*I 478 bp fragment of *Sox6* cDNA, located in the 5′ end of the coding sequence, specifically hybridized with the short and long transcripts of *Sox6*. The *aggrecan* probe was a 650 bp fragment of the mouse cDNA, encoding the C-terminal half of the G2 domain and most of the KS adjacent domain (Walcz *et al.*, 1994). Other probes were as described previously (Lefebvre *et al.*, 1995, 1997).

Northern blotting

Total RNA was isolated and analyzed in Northern blots as described previously (Lefebvre *et al.*, 1997). To facilitate transfer of large RNA species to nylon membrane, agarose gels were treated with 50 mM NaOH for 15 min before blotting. RNA standards were from Gibco-BRL. Hybridization signals were quantified on autoradiograms using the Intelligent Quantifier software program from Bio Image (Ann Arbor, MI).

In situ hybridization

Preparation of mouse embryo sections and hybridization with sense and antisense RNA probes labeled with [α-^{35}S]UTP were performed as described previously (Zhao *et al.*, 1997). Autoradiograms with collagen and Sox RNA probes were developed after 1 and 6–7 days, respectively. Sense probes showed no detectable signal over background.

Expression plasmids

Full-length mouse L-Sox5 and Sox6 coding sequences and deletions 151/679 and 213/679 were amplified by PCR and cloned into the pcDNA–5′UT and pcDNA–5′UT-FLAG mammalian expression plasmids, as described previously for human SOX9 and mouse Sox5 and Sox4

(Lefebvre *et al.*, 1997). The FLAG epitope did not affect DNA binding and transactivation properties of any Sox protein (data not shown). PCR products were verified by DNA sequencing. To obtain deletion 32/437, an *Nco*I fragment of L-Sox5 cDNA was blunted and cloned into blunt-ended *Bam*HI and *Eco*RI sites of pcDNA–5′UT–FLAG; a translation termination codon was located in the *Xba*I site located downstream of the *Eco*RI site. Deletion 32/221–304/437 in L5 was obtained by cutting off a *Pst*I fragment in deletion 32/437. Sox6B (previously called SoxLZ) cDNA was a gift from Tadayoshi Shiba and Shinya Yamashita (Takamatsu *et al.*, 1995). Sox6A and Sox6C coding sequences were reconstituted by replacing the *Hin*cII fragment of *Sox6B* cDNA with *Sox6A*- and *Sox6C*-specific fragments, which were obtained by PCR.

Synthesis of protein in vitro and cross-linking

Protein was synthesized *in vitro* with [^{35}S]methionine. For cross-linking, 2 μl of protein sample was preincubated for 30 min in 7 μl of DNA-binding buffer, with or without 5 fmol of 2HMG oligonucleotide, and incubated for 10 min with 1 μl of 0.1% glutaraldehyde. Polypeptide species were separated by SDS–PAGE in a polyacrylamide gradient (5–12%) gel and revealed by autoradiography.

Cell cultures

All cell types were cultured as described previously (Lefebvre *et al.*, 1997). Primary chondrocytes from ribs of newborn mice were used, unless otherwise indicated, after 2–3 days in culture, when they were essentially fully differentiated (Lefebvre *et al.*, 1994). RCS cells were previously shown to display a highly stable phenotype of early-stage chondrocytes (Mukhopadhyay *et al.*, 1995). MC615 cells were used after early passage when they exhibited a fairly differentiated chondrocytic phenotype (Mallein-Gerin *et al.*, 1993) or after repeated passages when they had essentially lost their chondrocytic phenotype. Human recombinant bone morphogenetic protein-2 (Genetics Institute, Cambridge, MA) was added to the culture medium at a concentration of 150 ng/ml.

Transient transfection

The p309 *Col2a1*–βgeo reporter construct has been described previously (Zhou *et al.*, 1995). The βgeo gene encodes a fusion protein with *E.coli* β-galactosidase and neomycin-resistance activities. Constructs p309–(4x48), p309–(2x231) and p309–(2x221) were obtained by cloning wild-type and mutant enhancer fragments (Lefebvre *et al.*, 1996) into p309 as described previously for other constructs (Zhou *et al.*, 1995). Reporter constructs were cotransfected with Sox protein expression plasmids using lipofectamine (Gibco-BRL) (Lefebvre *et al.*, 1997). A plasmid, pSV2βgal or pGL2 (Promega), was included in all transfections as an internal control for transfection efficiency. Reporter activities were determined after normalization for transfection efficiency. Reporter and control plasmids were transfected in a 3:1 ratio, and expression plasmids were included in various amounts, as indicated. Empty expression plasmid was added, whenever necessary, to transfect the same total amount of DNA in all samples. To assess expression of endogenous genes, cells were transfected using either lipofectamine or FuGENE 6 (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions.

DDBJ/EMBL/GenBank accession numbers

The accession Nos are: L-Sox5, AJ010604; Sox6, AJ010605.

Nomenclature

SOX refers to human proteins; Sox refers to mouse proteins. Genes are italicized.

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